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Characterization of the Regulatory Elements of the Dopa Decarboxylase Gene in *Drosophila melanogaster*

by

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Li Chen

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Abstract

During Drosophila metamorphosis, the "early-late" genes constitute a unique class regulated by the steroid hormone 20-hydroxyecdysone. Their induction is comprised of both a primary and a secondary response to ecdysone. Previous work has suggested that the epidermal expression profile of the dopa decarboxylase gene (*Ddc*) is likely that of a typical early-late gene. The induction is a receptor-mediated primary response to ecdysone. *Ddc* activity is also dependent on the *Broad-Complex (BR-C)*. This thesis reports that the *BR-C* regulates *Ddc* expression at two different developmental stages through two different *cis*-acting regions. At pupariation, a single functional ecdysone response element, located at position –97 to –83 bp relative to the *Ddc* transcription initiation site mediates the direct response to ecdysone; the *BR-C* acts synergistically with the ecdysone receptor to up-regulate *Ddc*. DNAase I footprinting has identified four binding sites of the predominant *BR-C* Z2 isoform within a distal regulatory element that is required for maximal *Ddc* activity. The sites share a conserved core sequence with a set of BR-C sites that had been mapped previously to the first *Ddc* intron. Deletion of the intronic BR-C binding sites results in loss of *Ddc* activity at eclosion, suggesting that the *BR-C* is also involved in regulating target gene expression at the end of metamorphosis, perhaps independently of the ecdysone axis. At both pupariation and eclosion, the *BR-C* releases *Ddc* from active silencing mechanisms that operate through distinct *cis*-acting regions of the *Ddc* genomic domain. The Drosophila orphan receptor DHR38 binds alone to a conserved response element located within the 5' upstream silencing domain. One of its multiple isoforms functions as a repressor to inhibit

premature *Ddc* expression in the larval epidermal tissue and most importantly in the imaginal discs prior to the sclerotization pathway being initiated. Thus, both positive and negative regulatory mechanisms regulate *Ddc* expression to ensure that cellular and tissue morphogenesis is unencumbered by the overlying cuticle that is inappropriately sclerotized during Drosophila development.

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Abbreviations

ABC	ATP-binding cassette
br	*broad* allele
BR-C	*Broad-Complex* gene
CCAP	crustacean cardioactive peptide
COUP-TE	chicken ovalbumin upstream transcription factor
Ddc	*dopa decarboxylase* gene
DDC	dopa decarboxylase protein
DHR38	*hormone receptor 38* gene
DR	direct repeat
EcRE	ecdysone response elements
EH	eclosion hormone
EMSA	electrophoretic mobility shift assay
ETH	ecdysis-triggering hormone
JH	juvenile hormone
NBRE	NGFI-B response element
NGFI-B	nerve growth factor-induced protein B
npr1	*non-pupariating* allele
PA	pharate adults
PPAR	peroxisome proliferator-activated receptor
PTTH	prothoracicotrophic hormone
RAR	retinoic acid receptor

rbp	*reduced bristles on the palpus* allele
RT-PCR	reverse transcription-polymerase chain reaction
RXR	retinoid-X receptor
Svp	*Seven-up* gene
TR	thyroid hormone receptor
USP	*ultraspiracle* protein
VDR	vitamin D receptor
YPP	yolk protein precursor

Chapter 1

General introduction

Steroids, retinoids, and thyroid hormone function as critical signals to coordinate the growth, development, and physiology of higher organisms. Increases in hormone titer are transduced into changes in gene expression by ligand-dependent transcription factors that comprise the nuclear receptor superfamily (Mangelsdorf et al., 1995). Extensive molecular studies have identified over 150 members of this superfamily, most of which are designated as orphan receptors since they have no known hormone ligand. In-depth knowledge of Drosophila genetics and the high degree of interrelatedness of the human and fly genomes (Kornberg and Krasnow, 2000) make the fruit fly an ideal model system for studying the molecular mechanisms of steroid hormone action as well as the biological functions of orphan receptors during development.

In response to a neuropeptide, prothoracicotrophic hormone (PTTH), the Drosophila prothoracic gland secretes α–ecdysone into the circulatory system where it is modified by peripheral tissues into the physiologically active form of the hormone, 20-hydroxyecdysone (herein referred to as ecdysone) (Riddiford, 1993). Ecdysone functions as a critical temporal signal that drives the animal through its major postembryonic developmental transitions. The ecdysone titer shown in Fig. 1.1 shows definite peaks at about 8-10 hours of embryogenesis, and near the mid-point of the first and second larval instars. During the final larval instar, small ecdysone fluctuations may be important for timing developmental events. A small rise is seen 12 hours after ecdysis to the third instar, and about 12 hours later another small increase occurs just before the larvae leave the food. This ecdysone peak is presumably responsible for initiating the wandering behavior. A high titer pulse of ecdysone at the end of the third instar triggers puparium formation, marking the onset of the prepupal stage. Prepupal development proceeds for 12 hr, after which an ecdysone pulse initiates pupal development that lasts 3.5 days. Mid-way through this stage, the final peak of ecdysone occurs, followed by eclosion of the adult fly. Ecdysone affects virtually all tissues during metamorphosis, as the entire organism is reorganized; most larval tissues undergo a carefully orchestrated programmed cell death as the imaginal tissues take their place to form the adult insect. A focus of the current study is to understand how the systemic hormonal signal is refined into the appropriate stage- and tissue-specific developmental pathways that direct this transformation.

The first insights into the mechanisms by which ecdysone regulates metamorphosis came from the observation of the puffing patterns presented on the giant polytene chromosomes in the larval salivary glands (Ashburner, 1972; Clever and Romball, 1966; Thummel, 1996). These puffs represent transcribed genes. Approximately 15 'intermolt' puffs are present when the polytene chromosomes first become large enough to analyze in third instar. These puffs regress in response to the ecdysone pulse that triggers puparium formation. At least six 'early' puffs are rapidly induced. They regress after several hours, followed by the appearance of more than 100 'late' puffs. A few 'mid-prepupal' puffs are induced as the ecdysone titer drops after puparium formation, after which the prepupal ecdysone pulse re-induces the early-late puffing sequence. Through a series of detailed and elegant studies on the puffing patterns, Ashburner and co-workers proposed a model for the regulation of gene expression by ecdysone (Ashburner et al., 1974). Briefly, this model proposed that ecdysone, bound to its specific receptor, directly induces the expression of early puff genes, but represses the late genes. As the concentration of an early gene product increases with time, this displaces the ecdysone/receptor complex from both early and late genes, repressing the former and activating the latter. It was assumed that these late genes would function as effectors that directly or indirectly control the appropriate biological responses to the pulse of ecdysone. The late genes could be further divided into two classes (Ashburner and Richards, 1976). The early-late genes are induced relatively rapidly compared to the late-late genes and require the continuous presence of ecdysone for their activity, much like the early genes. A schematic representation of the Ashburner model is depicted in Fig. 1.2. In this introduction, I will summarize the recent studies on these ecdysone-inducible genes and provide an overview of how they expand our understanding of ecdysone function during development.

I. The ecdysone receptor.

The ecdysone receptor belongs to the nuclear receptor superfamily (Mangelsdorf et al., 1995). A typical nuclear receptor contains a variable N-terminal region (the A/B domain) which carries part of the transactivation function (AF-1); a strongly conserved DNA binding C domain, which is the signature of the family; a variable hinge region (domain D); and finally a less conserved E domain, which carries out several important functions of the receptor-- ligand binding, dimerization and transcriptional regulation (AF-2). The ecdysone receptor is the only ligand-dependent nuclear receptor identified to date in Drosophila (Table 1). It is a heterodimer that more closely resembles a vertebrate retinoic acid receptor than a steroid receptor (see below). One polypeptide of the receptor is encoded by the *EcR* gene, the Drosophila homolog of the farnesoid X receptor (Mangelsdorf et al., 1995). *EcR* is induced directly at the beginning of the gene activation hierarchy (Andres et al., 1993). EcR exists in three isoforms, designated EcR-A, B1 and B2 (Talbot et al., 1993). These proteins differ in their amino-terminal sequences but contain identical DNA binding and ligand binding domains. Interestingly, the EcR-B1 isoform is expressed primarily in larval cells that are fated to die, while EcR-A is expressed in developing adult structures. The biological activities of the retinoids are mediated by two nuclear hormone receptors: the RAR and the retinoid-X receptor (RXR). Likewise, each EcR isoform requires the protein Ultraspiracle (USP) as a partner in heterodimerization. USP is the Drosophila homolog of vertebrate RXR proteins (Thomas et al., 1993; Yao et al., 1992). Indeed, not only does USP share sequence identity with RXR, it can substitute for RXR by forming heterodimers with several known partners of RXR, including the thyroid hormone receptor, the vitamin D receptor, the retinoic acid receptor, and the peroxisome proliferator-activated receptor (Yao et al., 1992).

Although EcR can bind ecdysone on its own, binding is greatly stimulated by the addition of USP. Ligand binding further stabilizes the EcR-USP complex and increases its affinity for binding to ecdysone response elements (EcREs) in the promoters of genes (Yao et al., 1993). Recent evidence supports the idea that many nuclear receptors switch, in a ligand-dependent manner, between binding of a multi-component co-repressor complex containing histone deacetyltransferase activity, and binding of a co-activator complex containing factors with histone acetyltransferase activity (Torchia et al., 1998). Structural studies of a number of nuclear receptor ligand-binding domains (LBDs) have yielded a relatively simple mousetrap-like model for the mechanism by which ligands activate nuclear receptors (Moras and Gronemeyer, 1998). The LBDs of nuclear receptors share a common, predominantly helical, fold. Upon ligand binding, helix H11 is repositioned and H12 concomitantly flips over and forms a cap covering the cavity that accommodates ligand in the core of the LBD. Corepressors are released and the LBD adopts a unique conformation that generates an interation surface for the coactivators, which then recruit multiprotein complexes and lead to the activation of responsive genes. The RAR-RXR heterodimer binds to DNA recognition sites that are generally composed of a direct repeat of the half-site core motif PuGGTCA spaced by 2 (DR-2) or 5 (DR-5) base pairs (Predki et al., 1994). In contrast, the binding site for EcR-USP can be heterogeneous. It can be composed by directly repeated half-sites, like the *ng*-EcRE identified within the coding region of *ng-1* and *ng-2*, two highly homologous genes mapping at the ecdysone-regulated 3C intermolt puff (Antoniewski et al., 1996; Crispi et al., 1998). More often, EcREs are composed of degenerate palindromes, having a single undefined base at the center of symmetry as first reported for the *hsp27* and *Eip 28/29* genes (Cherbas et al., 1991; Riddihough and Pelham, 1987).

EcR and USP can also heterodimerize with other Drosophila receptors, thus increasing the spectrum of potential regulatory interactions. *Seven-up (Svp)*, the

Drosophila homolog of the chicken ovalbumin upstream transcription factor (COUP-TE) (Table 1), and *Usp* act antagonistically in the determination of the photoreceptor R7 cell fate (Zelhof et al., 1995). In a yeast two-hybrid system, *Svp* can interact with *EcR*, suggesting that SVP inhibits ecdysone signaling by formation of an inactive EcR-SVP heterodimer.

USP's second partner is hormone receptor 38 (DHR38, Table 1), a homolog of the rat nerve growth factor induced protein B (NGFI-B) (Fisk and Thummel, 1995). Transfection experiments in Schneider cells showed that DHR38 can compete with EcR for dimerization with USP and consequently disrupt ecdysone-dependent transcription (Sutherland et al., 1995). However, the best *in vivo* evidence came from the mosquito *Aedes aegypti* (Zhu et al., 2000). Co-immunoprecipitation experiments indicated that AaUSP associates with AHR38 instead of AaEcR in fat body nuclei at the state-of-arrest, preventing the activation of yolk protein precursor genes by ecdysone.

In the absence of ligand, the EcR/USP heterodimer can act as a repressor (Schubiger and Truman, 2000). Early metamorphic events in the wing, including neurogenesis and axonal outgrowth, clearly require ecdysone in order to occur, but this requirement is carried out through an ecdysone-dependent release of USP-mediated suppression. In the absence of USP, these events occur in a steroid-independent fashion.

To date, two Drosophila corepressors mediating transcriptional silencing of the EcR/USP heterodimer have been identified. SMRTER, related to the vertebrate corepressors SMRT and N-CoR, directly interacts with EcR (Tsai et al., 1999).

SMRTER also associates with Drosophila Sin3A, a co-repressor known to form a complex with the histone deacetylase Rpd3/HDAC. Alien, another corepressor that interacts with EcR directly (Dressel et al., 1999), also shows a strong association with Sin3A. All these data indicate a conserved mechanism underlying transcriptional repression by vertebrate and invertebrate nuclear receptors.

II. Early genes.

Early genes are defined as those that react by puffing within minutes of exposure to ecdysone and the response of the early puffs is independent of protein synthesis (Ashburner, 1972). However, inhibiting protein synthesis does have a consequence for the early puffs. Normally, the activities of these puffs are transient. In the continuous presence of ecdysone they reach a maximum size after about 4 hr and then regress. If protein synthesis is inhibited, this regression does not occur. This immediately suggested that these puffs are auto-regulated and that their regression is a consequence of their activity.

1. *E74, E75,* and *Broad Complex.*

These three early genes were the first to be cloned and they all encode ecdysone-inducible DNA-binding proteins, consistent with the prediction of the Ashburner model. Further molecular and genetic analysis has revealed that these early genes provide critical regulatory functions during metamorphosis. Both *E74* and *BR-C* encoded transcription factors have been shown to regulate gene expression through direct binding to DNA sequences in secondary response genes (Dubrovsky et al., 2001; Urness and Thummel, 1990). Interestingly, all three genes encode multiple isoforms. The *BR-C* encodes a family of four isoforms, each containing one of four possible pairs of zinc fingers, designated Z1-Z4 (DiBello et al., 1991). The E74A and E74B proteins share an identical ETS DNA-binding domain (Burtis et al., 1990); and the E75A, E75B and E75C proteins are orphan members of the nuclear hormone receptor superfamily (Segraves and Hogness, 1990). Although polytene chromosome puffs are seen easily only in the larval salivary glands, these genes are widely expressed during development. E74A transcription is induced in a variety of ecdysone target tissues in late third instar larvae and during each of the ecdysone pulses that mark the six stages of Drosophila development (Thummel, 1990). All tissues studied to date contain all of the BR-C isoforms. However, their relative abundance differs greatly among tissue types (Bayer et al., 1996). At puparium formation, a dramatic shift occurs in isoform expression from Z2 to Z1 (Emery et al., 1994). These observations lead to an extension of the Ashburner model to other tissues, which proposes that overlapping combinations of early transcription factors specify stage- and tissue-specific developmental responses through the regulation of distinct sets of secondary response genes.

2. *E63*. The early puff at 63F is significantly different from the above early puff loci. First, the E63-1 gene does not encode a transcriptional regulator. In contrast, it encodes a calcium-binding protein with four EF hands, most closely related to calmodulin (Andres and Thummel, 1995). Second, its induction in late third instar larvae appears to be highly tissue-specific, as it is restricted to the salivary gland. This has led to the proposal that *E63-1* might direct glue secretion in response to ecdysone. However, an *E63* null mutation has no apparent effect on development or fertility, possibly due to functional redundancy in this pathway (Vaskova et al., 2000).

3. *E23.*

The cloning of the *E23* early puff gene confirms that yet another non-transcriptional regulator is encoded by an early puff gene. This gene encodes a protein with homology to ATP-binding cassette (ABC) transporters, raising the possibility of a previously unrecognized regulatory mechanism for modulating steroid hormone signaling (Hock et al., 2000). Ectopic expression of *E23* in late third instar larvae leads to complete lethality, and the activation of a number of early-response genes, including *E74* and *E75*, is blocked. Thus, *E23* is acting as a negative regulator of hormone responses, fulfilling the requirement in the Ashburner model that at least one of the early puff proteins negatively regulates early gene expression (Fig. 1.2). One intriguing hypothesis is that ectopic *E23* reduces the effective concentration of ecdysone within the cell. This hypothesis is supported by data demonstrating that ABC transporters are capable of transporting steroids in a variety of organisms.

III. Early-late genes.

The early-late genes are a distinct class of early genes that requires both ecdysone and early protein synthesis for maximal induction. As the full induction of these genes is inhibited by cycloheximide they were originally considered to be a subclass of the late class of puffs whose appearance is likewise inhibited by cycloheximide (Ashburner et al., 1974). However, the early-late transcripts are directly induced by ecdysone and are distinguishable from the early transcripts only by their rate of accumulation. Thus the early-late transcripts are hierarchically equivalent to the early transcripts (Huet et al., 1995).

1. *E78*.

This gene encodes two nested transcription units, *E78A* and *E78B* (Stone and Thummel, 1993). *E78A* mRNA is expressed during a brief interval in midpupal development and encodes a novel member of the nuclear hormone receptor superfamily (Table 1). *E78B* encodes a truncated receptor isoform that lacks the DNA-binding domain and is predominantly expressed at puparium formation and immediately following *E78A* in pupae. *E78B* is directly inducible by ecdysone in late third instar larvae and depends on ecdysone-induced protein synthesis for its maximal level of expression. Mutant analysis shows that the function of both protein isoforms is completely dispensable for normal development (Russell et al., 1996). *E78B* seems to play a subtle modulating role in regulating a subset of late puffs (*63E* and *82F*) since these puffs are reduced in size in *E78B* mutant background.

2. *Hormone receptor-like in 46*.

This gene also encodes an orphan nuclear receptor, *DHR3* (Koelle et al., 1992). *DHR3* is expressed in parallel with ecdysone pulses throughout development and is induced directly by ecdysone in late-third-instar larvae. Like other early-late genes, its peak expression is delayed relative to that of the early genes. As a result of this delay, *DHR3* is expressed in early prepupae, as the early genes are repressed and *βFTZ-F1* is induced. *βFTZ-F1* encodes another orphan member of the nuclear receptor superfamily, related to the vertebrate SF-1 receptor (Lavorgna et al., 1993). *βFTZ-F1* is expressed during a brief interval in mid-prepupae, immediately preceding the re-induction of the early genes by the prepupal pulse of ecdysone. Gain-of-function and loss-of-function genetic studies have demonstrated that *βFTZ-F1* is a critical regulator of the prepupal–pupal transition (Broadus et al., 1999; Woodard et al., 1994). *βFTZ-F1* is both necessary and sufficient for maximal re-induction of early gene transcription in prepupae, as a critical competence factor that determines the appropriate genetic and biological responses to the prepupal pulse of ecdysone.

DHR3 is an essential regulator of the *βFTZ-F1* mid-prepupal competence factor, providing a functional link between the late larval and prepupal responses to ecdysone. Induction of *DHR3* in early prepupae ensures that responses to the prepupal ecdysone pulse will be distinct from responses to the late larval pulse, so that the animal progresses in an appropriate manner through the early stages of metamorphosis. The timing of the developmental events leading to the appearance of βFTZ-F1 is summarized in Fig. 1.3. βFTZ-F1 is not produced as soon as *DHR3* is expressed but only when E75B levels plummet. Unlike *E75A* which encodes a nuclear receptor with the two zinc fingers typical of a nuclear receptor DNA binding domain, *E75B* encodes an aberrant receptor that contains only the second finger (Segraves, 1990). Thus, E75B itself can not bind to DNA. However, by forming a complex with DHR3 on the *βFTZ-F1* promoter, E75B inhibits the *βFTZ-F1* induction by *DHR3* (White et al., 1997). The rapid disappearance of E75B after pupariation formation provides the timing mechanism for *βFTZ-F1* induction.

3. *D-spinophilin*.

Not all early puff genes function as direct transcription factors. Similarly, the 62E early-late puff does not encode a transcriptional regulator (Keegan et al., 2000). Denoted as *D-spinophilin*, it is the only homolog to the mammalian protein spinophilin/neurabin II. The D-spinophilin protein is predicted to contain a highly conserved PP1-binding domain and adjacent PDZ domain, as well as a coiled-coil domain and SAM domain, all of which can mediate protein-protein interactions. Members of the neurabin/spinophilin protein family act as multi-functional adaptors, tethering target proteins to each other and to the cytoskeleton. The dynamic expression of *D-spinophilin* in the developing CNS during metamorphosis raises the interesting possibility that *D-spinophilin* may mediate the cytoskeletal changes associated with the dramatic remodeling of the CNS during metamorphosis.

4. *Ddc*

The *Ddc* gene is the sole structural gene for the enzyme dopa decarboxylase (DDC) in the *Drosophila melanogaster* genome (Hirsh and Davidson, 1981; Hodgetts, 1975; Wright et al., 1976). DDC catalyzes the decarboxylation of dopa to dopamine and 5-hydroxytryptophan to serotonin (Clark et al., 1978; Livingston and Tempel, 1983; Wright et al., 1976). Dopamine and serotonin serve two known physiological functions in Drosophila. Dopamine metabolites serve as crosslinking agents for cuticular proteins during sclerotization, while both dopamine and serotonin serve as neurotransmitters. DDC is a vital enzyme, which is expressed in a stage- and tissue-specific manner (Konrad and Marsh, 1987; Kraminsky et al., 1980). The *Ddc* primary transcript is expressed primarily in the central nervous system (CNS) and the hypoderm, where *Ddc* is alternatively spliced into different mRNAs encoding distinct DDC isoforms (Eveleth et al., 1986; Morgan et al., 1986). The *Ddc* CNS-specific mRNA contains four exons (A through D), whereas the hypodermal mRNA contains only three (A, C, and D), skipping the second exon. In the CNS, *Ddc* is expressed only in about 150 dopamine and serotonin neurons and in a subset of glial cells (Beall and Hirsh, 1987; Konrad and Marsh, 1987). Epidermal DDC is found within the epidermal cells and is not detected in the overlying cuticle. The level of *Ddc* activity remains relatively constant in the CNS throughout development. The precise pattern of neuronal *Ddc* expression requires at least two regulatory regions, a binding site for the factor *Elf1* at -60 bp and a binding site for the *Cf1* at around -600 bp (Bray et al., 1988; Johnson and Hirsh, 1990). Furthermore, glial expression of the *Ddc* gene is repressed by a less defined regulatory region located approximately 1kb upstream of the transcriptional start site (Mastick and Scholnick, 1992). Peaks of activity are observed at each of the five molts in the epidermis (Kraminsky et al., 1980). Transcripts of the *Ddc* gene accumulate within 4 hr of the administration of ecdysone to mature larvae of the temperature sensitive ecdysone-less mutant, *ecd*^{1} (Kraminsky et al., 1980). The increase can be explained as the sum of a direct steroid effect independent of protein synthesis, and an indirect effect dependent on proteins synthesized after an increase in the hormone titer (Clark et al., 1986). The observation that mutations in the *broad* sub-complementation group of the *BR-C* locus reduce *Ddc* mRNA levels and enzyme activity at pupariation (Hodgetts et al., 1995; O'Keefe et al., 1995) implicated the *BR-C* as the secondary response component of *Ddc* induction.

IV. Late-late genes.

1. *L71*.

As outlined above, most recent studies of the metamorphic ecdysone response in *D. melanogaster* have focused on the regulatory genes at the top of the ecdysone hierarchy. Comparatively little is known about the late puffs and the genes they encode. Of the more than 100 late-late puffs present in the larval salivary gland polytene chromosomes, only one puff had been characterized at the molecular level prior to 1999. The 71E cytogenetic region contains a cluster of five pairs of very short (~450 bp) divergently transcribed late genes (Restifo and Guild, 1986). Consistent with the Ashburner model, these genes are only expressed in one tissue and they require the activity of the early genes such as the *Broad-Complex* and *E74* for transcriptional activation (Crossgrove et al., 1996; Urness and Thummel, 1995). Although no function has yet been ascribed to the *L71* genes, they encode a family of secreted, basic proteins that resemble vertebrate defensins (Wright et al., 1996). In mammals, epithelial defensins are produced in the mucosal cells lining the lumen of the small bowel and may constitute an antimicrobial host defense system (Jones and Bevins, 1993). It seems possible that secretion of the L71 proteins from the salivary glands into the space between the imaginal hypoderm and prepupal cuticle may protect the vulnerable metamorphosing animal from infection.

2. *L63 and L82.*

The paucity of data concerning the late genes is exemplified by the fact that the molecular genetic analysis of the three best characterized late puffs, at cytological positions 22C, 63E,and 82F (Ashburner and Richards, 1976) has only just begun for the 63E (Stowers et al., 2000) and 82F (Stowers et al., 1999) puffs, while the 22C puff has yet to be studied. The initial characterization of *L63* and *L82* genes clearly shows that these late puff genes most closely resemble early genes such as the *Broad-Complex* rather than the *L71* late genes. It seems that the notion that late genes encode simple effector functions of ecdysone signaling may be the exception rather than the rule. First, both genes are unusually long and complex. *L63* spans 85 kb of genomic DNA that includes three overlapping transcription units, which generate nine different mRNAs encoding five different *L63* isoforms. *L82* is ~53 kb in length and directs the synthesis of at least seven mRNA isoforms from distinct promoters.

Second, whereas *L71* gene expression is limited to the terminal stages of the larval salivary gland's existence, one or more of the *L63* and *L82* transcription units are expressed throughout Drosophila development and in many different tissues. Third, molecular and genetic analyses suggest that transcriptional regulation of both *L63* and *L82* has ecdysone-dependent and ecdysone-independent aspects. Although there is a correlation between the expression of some *L63* and *L82* mRNAs and the increase in ecdysone titer at the onset of metamorphosis, the majority of the transcription appears not to be under ecdysone control. Finally, while *L71* encodes proteins that are consistent with their being "effectors" of the ecdysone response, the proteins encoded by the *L63* and *L82* genes do not fit easily into this category. Sequence analysis of the L82 proteins reveals two conserved domains in the predicted protein isoforms, a short prokaryotic homology region found in the L82A and L82B isoforms and a longer eukaryotic homology region found in all L82 isoforms. Although the functions of these domains remain unknown, *L82* appears to be an essential gene. The *L82* mutations cause developmental delays, with null alleles doubling the developmental time of the animals.

The L63 proteins contain a common C-terminal 294-aa sequence that is 71% identical to the CDK sequence of the murine PFTAIRE protein. *In vivo* tests of L63 proteins altered by site-directed mutagenesis showed that they exhibit CDK functions. However, there is no evidence that L63 is involved in the control of cell division as is the case for many CDKs. In fact, several observations suggest that, like PCTAIRE, L63 may play a role in cell differentiation rather than in cell division.

In summary, the Ashburner model has provided a durable framework for understanding the basic mechanisms of ecdysone-regulated gene cascades. However, as the characterization of genes at different levels in the hierarchy proceeds, it is clear that the model is oversimplified. Table 2 presents a synopsis of the three classes of genes discussed in this chapter. The diversity of proteins encoded by the hormone regulated genes goes far beyond the simple predictions of the model. Yet the ability to describe the expression of all these genes within a slightly revised version of the original model is a testimony to the insight of those who conceived it.

This thesis focuses on the molecular mechanisms that control *Ddc* expression in the epidermis, a representative of the early-late genes. The characterization of some of the necessary regulatory elements required to generate the normal *Ddc* expression pattern reveals that regulation of the *Ddc* gene in the epidermis involves both repression and activation, similar to its regulation in the CNS by both positive and negative mechanisms.

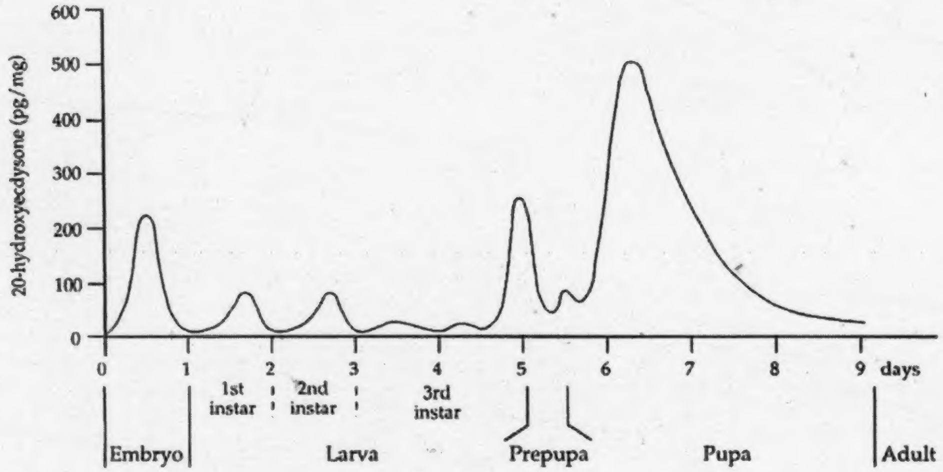

Figure 1.1. The ecdysone titer during Drosophila development. The composite ecdysteroid titers in *D. melanogaster* are presented in 20-hydroxyecdysone equivalents in whole body homogenates (figure adapted from Riddiford, 1993). Each of the developmental stages of the Drosophila life cycle is also shown, below a time-scale in days.

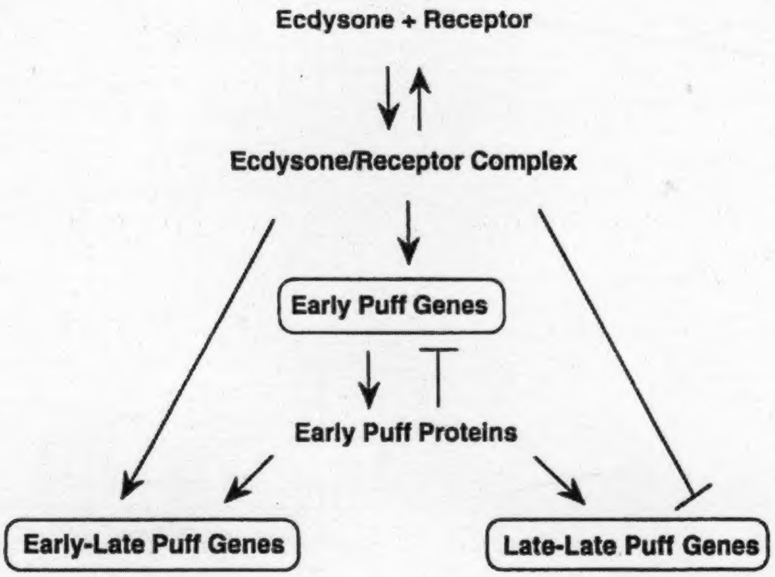
Ecdysone + Receptor
Ecdysone/Receptor Complex
Early Puff Genes
Early Puff Proteins
Early-Late Puff Genes
Late-Late Puff Genes

Figure 1.2. The Ashburner model for ecdysone-regulated puff gene expression (figure adapted from Ashburner et. al., 1974).

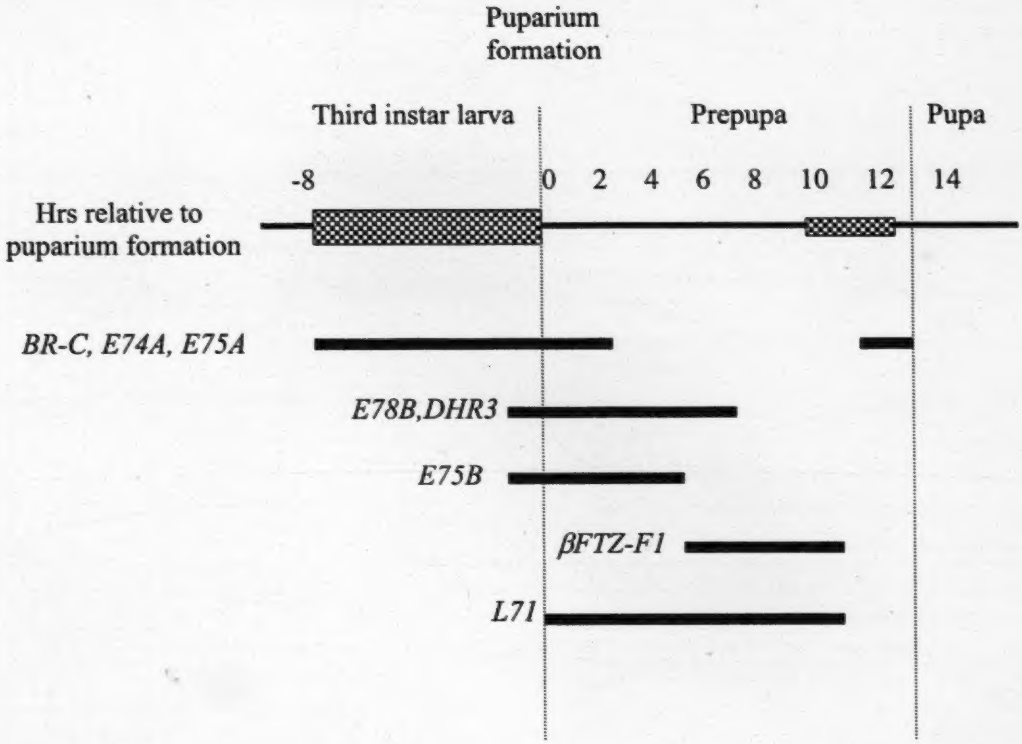

Figure 1.3. Schematic representation of ecdysone-regulated gene transcription during the onset of Drosophila metamorphosis. The late-third-instar larval, prepupal, and pupal stages of development are indicated at the top, above a timeline in hours relative to puparium formation. The late larval and prepupal ecdysone pulses are represented by hatched boxes, with the magnitude of each pulse represented by the width of the box. The timing of ecdysone-regulated gene transcription is shown by black boxes.

Table 1.1. Drosophila nuclear hormone receptor superfamily members (table adapted from Thummel, 1995).

Fly Receptor	Cytogenetic Location	Vertebrate Homolog	Ecdysone Regulation
KNI	77E		
KNRL	77E		
EGON	79B		
SVP	87B	COUP-TF	
TII	100A	TLX	
dHNF4	29E	HNF4	
FTZ-F1	75D	SF1	+
USP	2C	RXR	-
EcR	42A	Farnesoid X receptor	+
E75	75B	Rev-Erb	+
E78	78C	Rev-Erb	+
DHR3	46F	RORa?	+
DHR38	38E	NGFI-B	-
DHR39	39C	SF1	+
DHR78	78D	TR2	+
DHR96	96B	Human vitamin D receptor	+

* + indicates transcriptional regulation by ecdysone in cultured larval organs, while - indicates no apparent response.

Table 1.2. Summary of ecdysone-regulated genes

Gene	Corresponding Puff	Protein Isoforms	Encoded Protein
Broad Complex	2B5 early	4	Zinc-finger protein with BTB/POZ domain
E74	74EF early	2	ETS protein
E75	75B early	3	Nuclear receptor
E23	23E early	1	ABC transporter
E63-1	63F early	2	Calcium-binding protein
E78	78C early-late	2	Nuclear receptor
DHR3	46F early-late	3	Nuclear receptor
Ddc	37C early-late	2	Decarboxylase
D-spinophilin	62E early-late	5	Spinophilin/neurabin II
L71	71E late-late	10	Defensins
L82	82F late-late	7	Unknown function
L63	63E late-late	5	CDK

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Chapter 2

Induction of the early-late *Ddc* gene during Drosophila metamorphosis by the ecdysone receptor ¶

INTRODUCTION

During metamorphosis in *Drosophila melanogaster*, obsolete larval tissues are either histolyzed or re-organized into adult structures, many of which develop from the clusters of progenitor cells known as imaginal discs. These diverse fates are triggered by pulses of a single developmental cue, the steroid 20-hydroxyecdysone, hereafter referred to as ecdysone (Thummel, 1996)). Detailed studies of the puffing patterns of the larval salivary gland polytene chromosomes form the basis of a model for ecdysone action proposed by Ashburner and his colleagues (Ashburner et al., 1974). In their model, the hormone complexed with a receptor, directly and rapidly induces at least a half dozen "early" puffs. The products of these early puff genes were postulated to encode regulatory proteins that both repress their own expression and induce a large set of secondary-response or "late" puffs by overriding their receptor-mediated repression. These late gene proteins comprise the tissue-specific response of the target cells to the steroid. Many features of this model have been confirmed by more recent molecular studies. The functional ecdysone receptor consists of two members of the *Drosophila* nuclear receptor family: EcR

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(Koelle, 1992) and USP (Yao et al., 1992), the *Drosophila* homologue of the retinoic X receptor (Oro et al., 1990). So far, five early genes have been characterized at the molecular level, the *Broad-Complex* (*BR-C*), *E23*, *E74*, *E75* and *E63-1* (Andres and Thummel, 1995; Burtis et al., 1990; DiBello et al., 1991; Hock et al., 2000; Segraves and Hogness, 1990). Consistent with their proposed regulatory function, *BR-C*, *E74* and *E75* all encode proteins structurally related to transcription factors. With the exception of E63-1, which is found exclusively in the salivary gland, the early gene products are found in most tissues of the organism, suggesting that the ecdysone hierarchy, described in the Ashburner model, operates in all the ecdysone target tissues.

Despite the fact that the main components transducing the ecdysone response have already been identified, little is known about the molecular mechanism by which this single hormone elicits so large a variety of temporal and spatial specific responses during development. It has been shown that *EcR* encodes three isoforms that have different tissue and stage expression profiles (Talbot et al., 1993), and that USP can be differentially phosphorylated (Song and Gilbert, 1998). Thus, at least part of the tissue- and stage-specific responses to ecdysone could be dictated by modulations in the expression and activity of the receptor proteins. The EcR/USP heterodimer activates transcription through ecdysone response elements (EcREs) (Cherbas et al., 1991; Riddihough and Pelham, 1987). These sites harbor a 15-bp imperfect palindrome composed of two heptameric half-sites separated by one central nucleotide. They present homology with inverted repeats of the motif

PuG(G/T)TCA, known to be target sites for vertebrate nuclear receptors. However, the palindromic binding sites admit large sequence variation and can be very degenerate. In fact, they can even be composed of directly repeated half-sites (Antoniewski et al., 1996; Crispi et al., 1998). This suggests the specificity of the hormonal response could also lie in a wide repertoire of EcRE elements having different properties.

Interestingly, the late gene class has been further differentiated into early-late and late genes on the basis of hormone withdrawal experiments (Ashburner and Richards, 1976). The early-late genes appear to share properties with both the early genes and late genes (Stone and Thummel, 1993). For their full induction, a primary response to ecdysone and a secondary response to an early gene product are required, evidenced by the finding that inhibition of protein synthesis in the presence of ecdysone reduces but does not eliminate their transcription. A good example of the early-late gene class in *Drosophila* is *Ddc*. The gene encodes dopa decarboxylase, an enzyme that catalyzes the decarboxylation of dopa to dopamine and 5-hydroxytryptophan to serotonin (Dewhurst et al., 1972; Livingston and Tempel, 1983; Wright et al., 1976). DDC is expressed in a stage- and tissue-specific manner (Beall and Hirsh, 1987; Konrad and Marsh, 1987; Kraminsky et al., 1980). Most of the DDC activity (>90%) is found in the epidermal cells where dopamine metabolites promote pigmentation and cross-linking of the cuticle. Some DDC activity (about 5%) is also found in the central nervous system where it produces dopamine and serotonin, which serve as neurotransmitters. The level of *Ddc* activity remains relatively constant in the CNS throughout development, but peaks of activity are observed at each of the five molts in the epidermis (Kraminsky et al., 1980). At pupariation, *Ddc* transcripts increase rapidly following the exposure of hormonally naïve epidermis to exogenous ecdysone. The increase can be explained as the sum of a direct steroid effect, independent of protein synthesis and an indirect effect, dependent on proteins synthesized after an increase in the hormone titer, since inhibitors of protein synthesis reduce *Ddc* levels substantially from control levels (Clark et al., 1986). The protein required for full *Ddc* induction is encoded by the *BR-C*. The *Broad-Complex* encodes four isoforms, Z1-Z4 (Bayer et al., 1996; DiBello et al., 1991). Genetically, the *BR-C* locus contains three complementation groups: *broad* (*br*), *reduced bristles on the palpus* (*rbp*), and *2Bc* as well as a group of *non-pupariating* (*npr1*) alleles that are phenotypically indistinguishable from deletions of the locus (Kiss et al., 1988). Although there is not a simple correlation between the *BR-C* proteins and genetic function as defined by the intragenic complementation pattern, it has been observed that the br^+ function is provided solely by the Z2 isoform (Bayer et al., 1997). The observation that mutations in the *br* sub-complementation group of the locus reduce *Ddc* mRNA levels and enzyme activity at pupariation (Hodgetts et al., 1995; O'Keefe et al., 1995) implicated the Z2 isoform in the secondary response component of *Ddc* induction.

In this paper, we focused on defining the molecular mechanism underlying the primary response component of *Ddc* induction in the epidermis of mature larvae. *Ddc* regulatory elements essential for proper expression in the larval epidermis have been mapped within the 5'-flanking region by deletion analysis (Beall and Hirsh, 1987; Hirsh et al., 1986; Mastick and Scholnick, 1992; Scholnick et al., 1983). These studies concluded that no sequences upstream of an *Eco*RV site at –215 bp (now repositioned at –208 bp) were required for proper epidermal expression. Downstream of –208 bp, two overlapping elements lying between –106 and –83 bp were shown to contribute significantly to induced levels of DDC at pupariation (Scholnick et al., 1986). Here we report that one of these, element III, comprises part of a functional EcRE that is necessary for the rapid early response to ecdysone. In addition, we present the first evidence that an element controlling epidermal *Ddc* lies upstream of –208 bp. Transgenic flies carrying variously deleted upstream regions allowed us to locate a silencing domain between –1427 and –2067 bp that represses *Ddc* until the time at which the newly secreted cuticle must be hardened.

RESULTS

Receptor binding to a putative EcRE.

We used DNAMAN (Lynnon BioSoft, Inc) to identify potential EcREs in the *Ddc* 5'-flanking region by their similarity to a consensus (Fig. 2.1A) derived from published EcRE sequences (Antoniewski et al., 1993; Cherbas et al., 1991; Vögtli et al., 1998). Among several other possible candidates, an imperfect palindrome, located between –97 and –83 bp relative to the *Ddc* transcription start was the best match to the consensus sequence (12 of 15 bases, Fig. 2.1A). We shall refer hereafter to this palindrome at position –97 as EcRE$_{-97}$. Between –97 and -83 bp are the overlapping homology elements IIB and III (Scholnick et al., 1986). Element III (underlined in Fig. 2.1A), a 9 bp sequence which contains a half-site of the putative

EcRE, is perfectly conserved between the *Ddc* genes of *D. melanogaster* and the distantly related species *D. virilis* (Scholnick et al., 1986). We also used a pair of *D. melanogaster* specific PCR primers to amplify the corresponding promoter region from *D. simulans*. Sequencing results showed that this 15-bp segment is perfectly conserved in *D. simulans*, despite many small changes between the motif and the transcription start site (Fig. 2.1B). Deletion of the interval between –106 and –83 bp significantly reduced hypodermal *Ddc* expression at pupariation (Scholnick et al., 1986). Hence, we set out to test the ability of EcRE_{-97} to bind the EcR/USP heterodimer using a gel mobility shift assay. The subunits of the EcR/USP were prepared by *in vitro* transcription/translation of cDNA clones of the *EcR-B1* and *usp* genes (Yao et al., 1992). As shown in Fig. 2.2, the formation of a specific complex with the palindromic *Ddc* probe was strictly dependent on the presence of both EcR and USP in the binding reaction (compare lanes 3 and 4 to lanes 5-7). Increasing amount of the complex was observed as the amount of receptor protein increased from 1 to 3 μl (lanes 5-7). Inclusion of a 50-fold excess of unlabelled EcRE_{-97} (lane 8) or the *hsp27* EcRE (lane 9) completely prevented the formation of a radio-labeled complex in the presence of 2 μl of receptor. The choice of this amount of protein to demonstrate the competition was arbitrary but since the probe is in excess, competition would be expected throughout the range of receptor amounts shown in the figure.

Ddc EcRE$_{-97}$ exhibits a lower binding affinity for the receptor than does the canonical hsp27 EcRE.

To characterize the binding capability of the EcRE$_{-97}$ for the ecdysone receptor complex, we compared its relative affinity with that of the *hsp27* EcRE. Varying concentrations of unlabelled, double-stranded EcRE oligonucleotides were tested for their ability to compete with a radio-labeled *hsp27* EcRE probe for binding to the receptor complex (Fig. 2.3). When the amount of radioactivity in the nucleo-protein complexes shown in lanes 1 and 2 of Fig. 2.3A was measured, we determined that a 25-fold excess of the specific unlabelled competitor reduced binding of the labeled oligonucleotide to 5% (Fig. 2.3B). As lanes 3-6 show, addition of a 100 to 200-fold excess of unlabelled *Ddc* EcRE$_{-97}$ was required to reduce binding to the 5% level. Under these conditions, a 200-fold excess of either a non-specific competitor (lane 7) or the mutated *Ddc* EcRE* shown in Fig. 2.1A (lane 8) produced no reduction in the binding. Taken together, the data in Fig. 2.3 indicate that the *Ddc* EcRE$_{-97}$ exhibits a 4 to 8-fold lower affinity *in vitro* than that of the *hsp27* EcRE for the receptor complex.

Positions +5 and +6 in EcRE$_{-97}$ are critical for receptor binding.

EcRE-97 differs from the EcRE consensus at positions +1, +5 and +6 (Fig. 2.1A). Any of these nucleotide changes could be responsible for the reduced receptor binding affinity. Since the base pair at position +1 of the *hsp27* EcRE palindrome can be mutated without effect on the EcR binding (Ozyhar and Pongs, 1993), the base pair (T/A) at this position does not seem to form contacts with the receptor. The divergence of EcRE$_{-97}$ from the consensus at position +5 was eliminated by substituting a C for the wild-type T in the *Ddc* EcRE$_{-97}$. The mutated sequence EcRE(+5) was used as a competitor in a binding experiment conducted with the *hsp27* EcRE as a probe (Fig. 2.4). As before, a 25-fold excess of the unlabelled *hsp27* oligonucleotide was sufficient to reduce the radiolabelled complex to 5% of its value in the absence of competitor (compare lanes 1 and 2), while 25% of the complex remained in the presence of a 100-fold excess of the *Ddc* EcRE$_{-97}$ (lane 3). In contrast, by the time a 100-fold excess of the either *Ddc* EcRE(+5) or *Ddc* EcRE(+6) was reached, complex formation had been reduced to about 5% (lanes 6 and 9). Since the (+5) (+6) double mutant exhibited virtually the same competitive ability as the *hsp 27* EcRE (data not shown), we conclude that the thymine and guanine at positions +5 and +6 in EcRE$_{-97}$ together are responsible for its reduced binding affinity relative to the *hsp27* element.

EcRE$_{-97}$ is necessary for the induction of Ddc by ecdysone.

To confirm that the binding of the ecdysone receptor complex to EcRE$_{-97}$ is functional *in vivo*, we tested the effects of disrupting it with two kinds of mutations. First, an internal deletion was made in the *Sca*I-*Hpa*I fragment (Fig. 2.5A) and the *Eco*RV-*Taq*I fragment (Fig. 2.5B) that removed the sequence between –97 and –83 bp. We recovered transgenic lines in which these altered upstream regions were used to drive *lacZ* expression. As shown in Fig. 2.6, deletion of the EcRE$_{-97}$ (lines P[*Ddc-lacZ*]SH-ΔEcRE) caused a drop in β-galactosidase induction at pupariation from almost 18-fold to less than 3-fold. We ascribe this small amount of residual induction to the involvement of the *Broad-Complex* in *Ddc* regulation. The *cis*-acting element through which *Broad-Complex* Z2 isoform acts has been mapped to a region upstream of the transcription start site between –1426 and –383 bp (see Chapter 3). As the figure shows, this three-fold increase in reporter activity seen over the period of early wandering was eliminated in transformed lines carrying P[*Ddc-lacZ*]ET-ΔEcRE, which are missing both components of the activating machinery. The nearly complete loss of induction seen in the receptor mutant (Fig. 2.1) may also be ascribed to the absence of both components of the activating machinery, due to the fact that the *BR-C* is ecdysone inducible. Because of the precocious induction of the reporter in all P[*Ddc-lacZ*]ET lines (see below), the level of induction seen over the period of wandering in the control lines carrying P[*Ddc-lacZ*]ET was significantly less than was observed for the endogenous *Ddc* gene during this time (data not shown).

The second disruption of the EcRE$_{-97}$ in the *Eco*RV-*Taq*I fragment involved introducing a cluster of five point mutations into its left half-site to create the mutated element, EcRE* (Fig. 2.1A). We did this because the deletion of the –97 to –83 bp region removed, not only the EcRE$_{-97}$, but also element IIB, which is required to repress *Ddc* expression in the glial cells (Scholnick et al., 1986). The five point mutations introduced into the left half site of EcRE$_{-97}$ did not change the sequence of element IIB and the remaining data in Fig. 2.6 show that lines carrying the plasmid P[*Ddc-lacZ*]ET-EcRE* likewise eliminated any induction during the wandering period.

Identification of a silencing element(s) whose loss results in premature Ddc expression.

From previous work on transformants carrying deletions within the 5'-*Ddc* region, it was concluded that no regulatory elements necessary for normal hypodermal expression were located upstream of position -208 bp relative to the *Ddc* transcription initiation site (Hirsh et al., 1986). However, when transformants carrying the reporter construct P[*Ddc-lacZ*]ET, in which *lacZ* expression was under the control of the *Eco*RV-*Taq*I fragment (extending from –208 to +839 bp) of the *Ddc* gene (Fig. 2.5B), were examined, six lines with different chromosomal locations of the transgene all exhibited strong epidermal expression in the early wandering stage (Fig. 2.7A and E). This was surprising in light of the fact that *Ddc* induction occurs near the end of wandering stage. The tissue specificity of this precocious expression was consistent with the tissue distribution of DDC seen in wild-type larvae. None of the lines showed reporter gene expression in muscle, fat tissue, salivary glands, gut or sex organs. The cuticle itself did not stain but the ordered array of epidermal cells attached to it expressed β-galactosidase activity. Although no detectable DDC activity is found in the wild-type larval discs before pupariation (Konrad and Marsh, 1987), surprisingly, all of the lines showed expression of the reporter in imaginal discs. The actual pattern varied considerably from line to line. The discs of P[*Ddc-lacZ*]ET1 (B-D) are similar to those in lines P[*Ddc-lacZ*]ET4 and P[*Ddc-lacZ*]ET6 (not shown). In line P[*Ddc-lacZ*]ET7, the eye disc behind the morphogenic furrow shows very strong *lacZ* expression (Fig. 2.7H) but in its other discs (wing and leg for example, Fig. 2.7F,G), the level of expression was similar to that seen in the lines just discussed. The precocious reporter activity seen in the discs of all these lines leads to the conclusion that the endogenous *Ddc* gene is silenced in epidermis and imaginal discs during the early third larval instar stage by an element(s) that lies outside of the *Eco*RV-*Taq*I fragment.

Further mapping of the silencer element.

Although a detailed understanding of the silencing mechanism awaits further study, we have defined a 5'-flanking region that is sufficient to mediate repression. Reporter constructs were made in which 2067 (*Sca*I), 1426 (*Bsm*I), or 382 (*Eco*RI) bp of upstream-flanking DNA was used to drive *lacZ* expression; all shared a common downstream endpoint at a +174 *Hpa*I site (Fig. 2.5A). The silencing element was mapped by assaying reporter activity in transformed lines carrying each of the deletion-bearing constructs. Whereas all four of the transformant lines carrying either P[*Ddc-lacZ*]RH or P[*Ddc-lacZ*]BH showed precocious β-galactosidase activity in both the larval epidermis (Fig. 2.8A,E) and in the imaginal discs (Fig. 2.8B-D and F-H), the four lines carrying P[*Ddc-lacZ*]SH exhibited very little hypodermal staining in early wandering larvae (Fig. 2.8I) and no disc staining (Fig. 2.8J-L). To further confirm that the *Sca*I-*Bsm*I region contains the silencing element, we subcloned this 640 bp fragment immediately upstream of the *Eco*RI-*Taq*I fragment (P[*Ddc-lacZ*]SH-ΔBR, Fig. 2.5A) to test whether it could still repress the premature expression. Histochemical assays on the three lines obtained exhibited very little hypodermal staining in early wandering larvae and no disc staining (data not shown). Thus, the silencing element is located between -2067 and –1427 bp.

DISCUSSION

In this chapter we present several lines of evidence for a direct role of the ecdysone receptor in *Ddc* induction. Biochemical and genetic data indicate that the EcR-B1/USP heterodimer directly up-regulates *Ddc* expression at the end of larval life through an imperfect ecdysone response element, EcRE$_{-97}$. Ecdysone-inducible genes are assigned to the early-late class based on their response to inhibitors of protein synthesis. All such genes exhibit some induction in the absence of protein synthesis, from which it has been inferred that they are direct targets of the ecdysone receptor. This inference is now confirmed by our data on EcRE$_{-97}$, the first ecdysone response element identified for an early-late gene in Drosophila. Its existence explains the induction of *Ddc* mRNA by ecdysone in the absence of protein synthesis (Clark et al., 1986), since the two components of the heterodimeric receptor, USP and EcR, are present before the increase in hormone titer that triggers *Ddc* induction (Andres et al., 1993).

To date, a limited number of EcREs have been characterized as both *in vitro* binding sites for the EcR/USP heterodimer and *in vivo* functional hormone response elements (Antoniewski et al., 1994; Antoniewski et al., 1995; Cherbas et al., 1991; Crispi et al., 1998; Lehmann and Korge, 1995; Riddihough and Pelham, 1987). Most of the EcREs identified so far, including *Ddc* EcRE$_{-97}$, are imperfect palindromes with a single intervening nucleotide. The palindromic EcREs are similar to the inverted repeats of the motif PuG(G/T)TCA, known to be target sites for vertebrate nuclear receptors (Mangelsdorf et al., 1995). In contrast to vertebrate palindromes, however, the EcRE palindromes are degenerate, highly asymmetric, response elements. The two half-sites contribute differently to the binding of the heterodimer components; in particular, the 5' half-site exhibits substantially higher affinity for both EcR and USP than the 3' half-site (Niedziela-Majka et al., 2000). Since USP exhibits approximately four-fold higher affinity for the 5' half-site than EcR, it has been suggested that USP may act as an anchor that preferentially binds the 5' half-site and allows the EcR/USP heterodimer to assume a defined orientation within the promoter region (Niedziela-Majka et al., 2000). This suggestion is supported by our finding that the cluster of point mutations introduced into the 5'-half site (EcRE*, Fig. 2.1A) eliminated its ability to bind the receptor (lane 8, Fig. 2.3) and produced the same effect on reporter expression as a deletion of the entire element (Fig. 2.6). The receptor-EcRE contact sites occur predominantly at the positions -7, -6, -5, -2, -1 and +2, +5, +6 of the palindromic sequence and the base pairs at positions -3, -4 and +1, +3, +4 of the palindrome can be mutated without effect on the receptor binding (Ozyhar and Pongs, 1993). The 5' half-site of EcRE$_{-97}$ matches the consensus perfectly but deviates from the consensus at positions +1, +5, and +6. The fact that positions +5 and +6 are contact sites with the receptor probably explains the reduced affinity of EcRE$_{-97}$ for the receptor that we noted (Fig. 2.3). In fact, a single base-pair substitution of the +5 T by the canonical C or the +6 G by the canonical T produced a roughly two-fold increase in the binding affinity of EcRE$_{-97}$ (Fig. 2.4).

The proximal *Ddc* regulatory region contains three elements that contribute to glial and epidermal expression. The two copies of an 11 bp glial-specific element, IIA and IIB, are required for activation and repression of *Ddc* respectively in these cells (Mastick and Scholnick, 1992). Element III, a 9 bp sequence that is conserved between *D. melanogaster* and the distantly related *D. virilis*, includes half of EcRE$_{-97}$. Although element III partially overlaps element IIB, the point mutations that we introduced into element III did not change the sequence of element IIB nor the spacing between any of these regulatory motifs. These mutations reduced epidermal *Ddc* expression to the same extent as the EcRE$_{-97}$ deletion (Fig. 2.6), which removed both elements IIB and III. This indicates that IIB is not involved in hypodermal *Ddc* activation.

The data in Fig. 2.6 indicate that despite two deviations from the consensus sequence, EcRE$_{-97}$ is a functional EcRE. This is expected from the exact sequence conservation of the response element between the two sibling species, *D. melanogaster* and *D. simulans*. The receptor's weak binding affinity for EcRE$_{-97}$ may explain why *Ddc* induction *in vivo* occurs at a high titer of ecdysone (Kraminsky et al., 1980) and requires Z2, a transcription factor encoded by *BR-C* (Hodgetts et al., 1995; O'Keefe et al., 1995). We propose that the high peak of DDC activity observed at pupariation is mediated by the combined action of the ecdysone receptor, bound to EcRE$_{-97}$, and the BR-C protein, bound to a different site in the upstream *Ddc* region (in preparation). We propose an interaction based on the fact that the BR-C contains a BTB/POZ domain in its N-terminal region (DiBello et al., 1991). This evolutionarily conserved motif can both activate (Kaplan and Calame, 1997; Kobayashi et al., 2000) or repress (Chang et al., 1996; Deltour et al., 1999; Deweindt et al., 1995; Seyfert et al., 1996) transcription through its ability to mediate protein-protein interactions. These are usually homomeric but can involve BTB regions from two different proteins (Bardwell and Treisman, 1994). The capacity for more extensive interactions is evident by the recruitment of members of the histone deacetylase complex (Dhordain et al., 1997; Grignani et al., 1998; Hong et al., 1997; Lin et al., 1998) and transcription factors (Pointud et al., 2001) to BTB domains. The crystal structure of the BTB domain from the human promyelocytic leukemia zinc finger (PLZF) protein reveals it to be a tightly intertwined dimer; however, an exposed groove is formed at the dimer interface "suggestive of a peptide binding site" (Ahmad et al., 1998). Since mutations in this charged pocket eliminated the repressive effects of its BTB domain on transcription (Melnick et al., 2000), it is clear that the conformation of the pocket plays a role in the transcriptional properties of complexes containing BTB proteins. The interaction between a *Drosophila* BTB-containing protein and a possible co-activator of transcription, dTAFII155 (Pointud et al., 2001) makes our hypothesis of a co-activator role for BR-C in transducing the ecdysone signal plausible.

Previous work has suggested that all the regulatory elements essential for normal tissue-specific and temporally regulated expression of the *Ddc* gene are located very close to or within the gene (Hirsh et al., 1986). Transgenes retaining only 208 bp of 5'-flanking sequences and 1 kb of 3'-flanking sequences appeared to be expressed normally. However, no transgenic line containing more than 800 bp of upstream flanking DNA was examined in this early work. The results in this paper show two regulatory domains controlling epidermal *Ddc* expression in mature larvae lie upstream of 800 bp. We have mapped a silencing element(s) between –2067 and –1427 bp. The *cis*-acting element through which BR-C acts has been mapped to a region upstream of the transcription start site between –1426 and –383 bp (see Chapter 3). The reason that transgenes truncated at –208 bp appeared to be expressed normally is likely that the loss of the silencer compensated for the loss of the *BR-C* activating domain. This would have resulted in premature expression of *Ddc* in the epidermis and the imaginal discs as was observed in Fig. 2.7. In virtually all of the early work, the determinations of *Ddc* activity were done on whole organisms, and the tissue distribution of the activity was not monitored. In line P[*Ddc-lacZ*]ET3, for example, we found that approximately 55% of the *Ddc*-driven reporter activity in the whole body was located in the imaginal discs. In fact, evidence of premature ectopic expression in the imaginal discs was present in the early work. Some *Ddc*$^{5'\Delta\text{-}209}$ strains, such as 24-16, had a significantly lower fraction of their total *Ddc* activity in the hypoderm than did the wild type (Hirsh et al., 1986). In addition, the observed over-expression of *Ddc* activity during embryogenesis in their report of the *Ddc*$^{5'\Delta\text{-}209}$ strain, 24-44, might have been caused by premature activation rather than by the effects of chromosomal position, as supposed.

The finding that the *Ddc* gene is actively repressed in both imaginal discs and the epidermis of third instar larvae is novel. In both cases, the repression is mediated by the same region of the upstream *Ddc* sequence, which suggests that a single trans-acting protein is involved in both tissues. Secretion by the imaginal discs of the chitin-containing pupal cuticle *in vivo* begins to occur 8 hr after pupariation (Fristrom and Liebrich, 1986), or about 14 hr after the ecdysone titer begins to increase in late third instar (Richards, 1980). Repression of *Ddc* in the imaginal discs insures that the sclerotization pathway is not activated before disc eversion and secretion of the pupal cuticle has occurred. This repression could be relieved by the appearance of the ligand-bound receptor, formed as the result of a small peak of ecdysone that occurs 12 hr after pupariation (Handler, 1982). Repression of *Ddc* in the epidermis of early wandering larvae likewise insures that tanning and hardening of the cuticle of the third instar larva does not occur until after the wandering stage. Again, release from the repressed state might be triggered by the ligand-bound receptor that could only form as the ecdysone levels begin to rise mid-way through wandering. Of course, the interesting question of why *Ddc* repression is not relieved in the imaginal discs in response to the elevated ecdysone titer that is present at pupariation is, at present, unanswered.

The identity of the repressor also remains to be worked out. Because we do not observe any precocious induction in lines carrying the P[*Ddc-lacZ*]SH-ΔEcRE construct (data not shown), we conclude the repressor does not require an intact EcRE, but functions entirely through the upstream silencing element. In the absence of the silencing element, we see precocious activity irrespective of whether or not an intact EcRE is present (data not shown). From this we conclude that the precocious expression is produced independently of the ecdysone axis. This allows us to test whether USP functions as the repressor. It has been shown that in the wing disc of Drosophila, genes that are normally expressed later, such as *β-Ftz-F1* and the Z1 isoform of the *Broad-Complex*, are expressed precociously in the absence of USP (Schubiger and Truman, 2000). These results suggest a model in which a USP homodimer or the unliganded heterodimeric receptor acts as a repressor. We used a *usp* cDNA under the control of a heat-inducible promoter to rescue *usp* mutants past their early lethal phase (Hall and Thummel, 1998). Rescued *usp* mutants fail to undergo puparium formation and deposit a supernumerary cuticle, indicating that a lack of *usp* function blocks the developmental responses to the ecdysone pulse at the end of the third instar. Crosses were made to generate *usp* and wild-type organisms carrying the P[*Ddc-lacZ*]SH reporter construct (Fig. 2.5A) and early third instar larvae were stained for β-gal activity. No apparent staining was observed in the epidermal tissue or in any of the larval imaginal discs (data not shown) from which we conclude that *usp* does not function as a repressor for *Ddc* transcription. However, it is possible that the role could be fulfilled by another nuclear receptor. One candidate is DHR38, which can heterodimerize with USP and silence transcription (Sutherland et al., 1995). Currently we are testing a number of orphan receptors, including DHR38, for their ability to bind to the silencing region.

MATERIALS AND METHODS

DNA sequencing.

Genomic DNA from *Drosophila simulans* was amplified using *Ddc*-specific primers from *Drosophila melanogaster* (5'-GGGAAGCACGTGAGCAGAAT-3', -398 to –379 bp; and 5'-CTTTTAAAGCCCGTCCAGCA-3', -25 to –44 bp). The PCR product was subcloned into pGEM-TEasy vector (Promega) and sequenced.

Plasmid construction.

All the plasmids that we used were derivatives of P[CaSpeR-AUG-β-gal] which carries a *lacZ* reporter gene in a translational fusion with a fragment from the *Adh* gene that excludes the promoter and transcription initiation site (Thummel et al., 1988). Fragments from the *Ddc* genomic region that included its transcription start site were cloned into this vector. The extent of the *Ddc* region driving *lacZ* expression in these vectors is shown in Fig. 2.5. First, the *Sca*I-*Hpa*I fragment (see Fig. 2.5A) was isolated and cloned into the *Eco*RV site of pBluescript SK(+) (Stratagene). It was released using the *Bam*HI and *Kpn*I sites in the polylinker and directionally subcloned into pP[CaSpeR-AUG-β-gal] to generate P[*Ddc-lacZ*]SH. The construct, P[*Ddc-lacZ*]RH, was made by cutting P[*Ddc-lacZ*]SH with *Eco*RI and re-circularizing the largest fragment. To make P[*Ddc-lacZ*]BH, the recombinant pBluescript SK(+) carrying the *Sca*I-*Hpa*I fragment was doubly digested with *Sma*I (which cuts in the polylinker) and *Bsm*I (which cuts the 5'-*Ddc* region at two closely spaced sites). The largest fragment was recovered, blunt ended with T4 DNA polymerase (Invitrogen) and re-circularized. The desired fragment was released from this plasmid as a *Bam*HI-*Kpn*I fragment and subcloned into P[CaSpeR-AUG-β-gal], generating P[*Ddc-lacZ*]BH. The reporter plasmid carrying *Bsm*I-*Eco*RI deletion within the *Sca*I-*Hpa*I fragment (P[*Ddc-lacZ*]SH-ΔBR, Fig. 2.5A) was created by inverse PCR using the *Sca*I-*Hpa*I insert in pBluescript SK(+) as the template and primers: 5'-AAGTATTTCTCCGCTTATAGG-3' (-1427 to –1446 bp) and 5'-GGAAGCACGTGAGCA-GAAT-3' (-398 to –379 bp).

The second set of constructs (Fig. 2.5B) all shared a common 5'-terminus at the *Eco*RV site (-208 bp). The *Ddc* fragment in P[*Ddc-lacZ*]ET was first cloned into the pGEM-T vector by PCR amplification of pDdc23, a plasmid containing the 7.6 kb *Pst*I genomic sequence known to be sufficient for proper developmental expression of *Ddc* (Scholnick et al., 1983). The two primers we used were 5'-CGGGATCCAATGCCTTTGATATCCAGTTAC-3' (forward) and 5'-CGGGATCCTCGAATGGTTAGAGCTAATATG-3' (reverse). *Bam*HI recognition sites (underlined in both primers) were included to facilitate subsequent removal of the fragment from pGEM-T and insertion into the P[CaSpeR-AUG-β-gal] reporter vector to create P[*Ddc-lacZ*]ET. A reporter construct containing a deletion of the presumptive EcRE element within this fragment (P[*Ddc-lacZ*]ET-ΔEcRE, Fig. 2.5B) was made using the above primer pair to amplify p148, a plasmid that contains a 5'-*Ddc* upstream region with an internal deletion from –97 to –83 bp (Mastick and Scholnick, 1992). P[*Ddc-lacZ*]SH-ΔEcRE (Fig. 2.5A) was made by replacing the *Eco*RV – *Nco*I fragment from P[*Ddc-lacZ*]SH with the *Eco*RV – *Nco*I fragment from P[*Ddc-lacZ*]ET-ΔEcRE. The reporter plasmid carrying point mutations within the EcRE (P[*Ddc-lacZ*]ET-EcRE*, Fig. 2.5B) was created by inverse PCR using the *Eco*RV-*Taq*I *Ddc* insert in pGEM-T as the template and primers: 5'-TTAAACGAATCGCAGCCGCTGTCGT-AAAAA-3' and 5'-CAGCGGCTGCGGACTGCGAT-3'. The sequence of the mutated element is underlined and replaced the sequence 5'-AACTT-3' found in the wild type. All constructs generated by PCR were confirmed by direct sequencing.

Gel mobility shift assay.

Receptor protein was generated *in vitro* from the expression vectors CMX-EcR-B1 and CMX-USP (Yao et al., 1992) that were used as templates for coupled transcription/translation in a rabbit reticulocyte lysate (Promega). One microgram of template was added to a final reaction volume of 50 μl according to the manufacturer's instructions. The translated proteins were incubated with binding buffer, which contained 100 mM KCl, 7.5% glycerol, 20 mM HEPES (pH 7.5), 2 mM dithiothreitol and 0.1% NP-40, on ice for 20 min in the presence of 2 μg of nonspecific competitor poly(dI-dC) and other oligonucleotide competitors as indicated in the text. Oligonucleotides used as either substrates or competitors in the binding reactions were obtained by annealing the single stranded sequences shown in Figure 2.2A to their complementary strands (Antoniewski et al., 1993). Their concentration was determined by staining with ethidium bromide and photographing under UV light (Sambrook et al., 1989). Approximately 1 ng of the ^{32}P end-labeled DNA fragment (10^8 cpm/μg) was added to the receptor protein mixture and the binding reaction was allowed to proceed at room temperature for 20 minutes. The reaction was then loaded into 4% non-denaturing polyacrylamide gel in 0.5X TBE running buffer. After electrophoresis, the gel was dried for autoradiography. Quantitative analysis of protein-DNA complexes was performed by cutting out the band from the gel and counting in an aqueous scintillation cocktail. The amount of binding was expressed as a percentage of the amount of bound probe detected in a gel slice from a lane onto which a control binding reaction, with no competitor added, was loaded.

Germline transformations.

Injections were carried out according to standard procedures (Rubin and Spradling, 1982) with slight modifications. Embryos (0-1 hr) of the *y w* genotype were collected from 2% agarose plates smeared with live yeast paste and then dechorionated for 45-60 s using 50% Javex bleach. A DNA mixture of 10 µg of each construct and 2 µg of the Δ2-3 helper vector was precipitated and dissolved in 10 µl of water, prior to injection. All the needles used for injections were pulled using a Sutter Instrument Co. Model P-87 Fleming/Brown micro-pipette puller.

Histochemical assays.

LacZ staining was carried out according to (Bellen et al., 1989) with slight modifications. The integument and the imaginal discs were dissected in PBS (140 mM NaCl, 2.6 mM KCL, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, pH 7.4), fixed in 0.75% glutaraldehyde for 15 min and then washed three times in PBT (PBS containing 0.05% Triton X-100). Staining solution (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 0.1% X-gal in N, N-dimethylformamide) was prepared fresh daily and then added to the dissected tissues and allowed to stain for 2 h at 37 °C. The tissues were then washed three times in PBT and mounted in 80% glycerol. To measure β-galactosidase activity on crude extracts, three to four organisms were homogenized in 0.15 ml of 50 mM potassium phosphate, pH 7.5, 1 mM $MgCl_2$, 0.01

mM DTT. The extracts were cleared by centrifugation. Reactions were carried out by incubating 25 µl of extract, 180 µl ONPG solution (0.4% ONPG in homogenization buffer) at 37°C for 60 min. The reactions were stopped by adding 0.6 ml of 1M Na_2CO_3. The optical density readings at 420 nm were compared with those produced by substituting 180 µl of homogenization buffer for the ONPG solution in control reactions.

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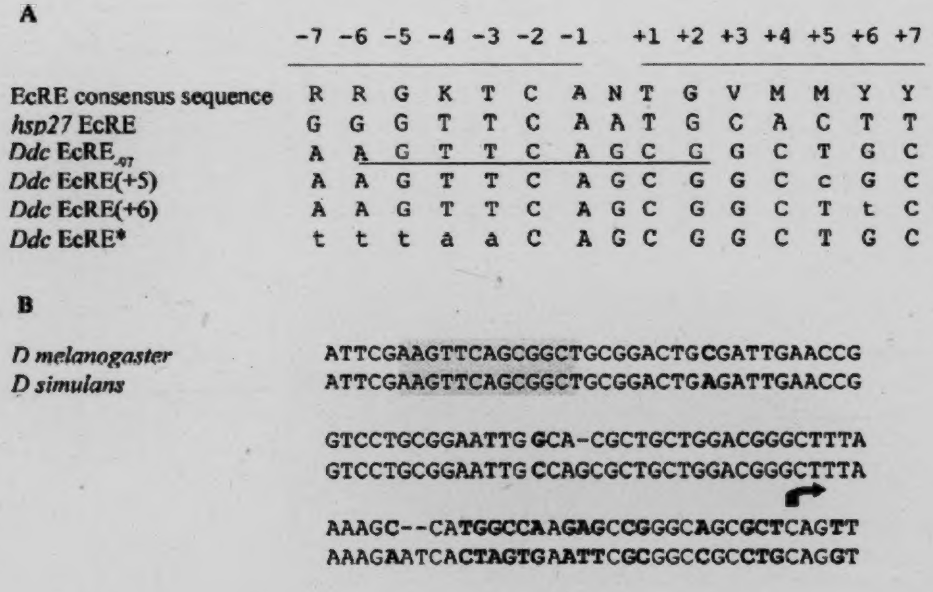

A

	-7	-6	-5	-4	-3	-2	-1		+1	+2	+3	+4	+5	+6	+7
EcRE consensus sequence	R	R	G	K	T	C	A	N	T	G	V	M	M	Y	Y
hsp27 EcRE	G	G	G	T	T	C	A	A	T	G	C	A	C	T	T
Ddc EcRE$_{-97}$	A	A	G	T	T	C	A	G	C	G	G	C	T	G	C
Ddc EcRE(+5)	A	A	G	T	T	C	A	G	C	G	G	C	c	G	C
Ddc EcRE(+6)	A	A	G	T	T	C	A	G	C	G	G	C	T	t	C
Ddc EcRE*	t	t	t	a	a	C	A	G	C	G	G	C	T	G	C

B

D melanogaster ATTCGAAGTTCAGCGGCTGCGGACTG**C**GATTGAACCG
D simulans ATTCGAAGTTCAGCGGCTGCGGACTG**A**GATTGAACCG

GTCCTGCGGAATTG **G**CA-CGCTGCTGGACGGGCTTTA
GTCCTGCGGAATTG **C**CAGCGCTGCTGGACGGGCTTTA

AAAGC--CA**TGGCCAAGAG**CC**G**GGC**A**GC**GCT**CAG**TT**
AAAG**AA**TCA**CTAGTG**A**ATT**C**GC**GGCCGC**CTG**CAG**GT**

Figure 2.1. A. Sequence comparisons between EcREs. *Ddc* sequence from –97 to –83 bp is compared to a consensus EcRE derived from previous work (Antoniewski et al., 1993; Cherbas et al., 1991; Vögtli et al., 1998), and to the *hsp27* EcRE (Riddihough and Pelham, 1987). Base positions within the palindromic repeat structure are indicated by lines above the consensus. R: purine; Y: pyrimidine; K: G or T; V: A or C or G; M: A or C; and N: any nucleotide. Element III (Scholnick et al., 1986) is underlined within the *Ddc* EcRE. Point mutations used in later experiments were introduced into the *Ddc* EcRE$_{-97}$ and are shown in small case. B. Genomic sequence comparison between *D. melanogaster* and *D. simulans* of the region containing EcRE$_{-97}$. The EcRE in both species is highlighted and mismatches are shown in bold case. The transcription start site from *D. melanogaster* (Morgan et al., 1986) is indicated with an arrow.

	1	2	3	4	5	6	7	8	9
EcR			+		+	++	+++	++	++
USP				+	+	++	+++	++	++
RT		+							
50× *Ddc* EcRE$_{-97}$								+	
50×*hsp27* EcRE									+

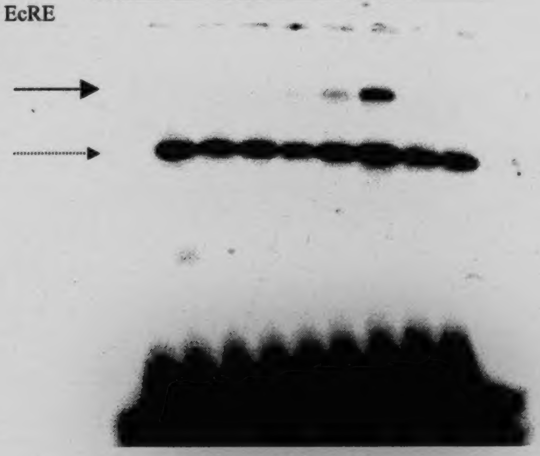

Figure 2.2. Band shifts reveal co-operative binding of EcR and USP proteins to the EcRE$_{-97}$ probe. A series of common bands (indicated by the dotted arrow) due to non-specific binding of a reticulocyte lysate protein (RT) to the ^{32}P-labelled probe is observed in all lanes. The specific EcR/USP complex is indicated by the solid arrow. Relative quantities of EcR and USP are indicated by + (1 μl), ++ (2 μl) or +++ (3 μl) of the same translation reaction. Unlabelled EcRE$_{-97}$ (lane 8) or the *hsp27* EcRE (lane 9) were added as specific competitors.

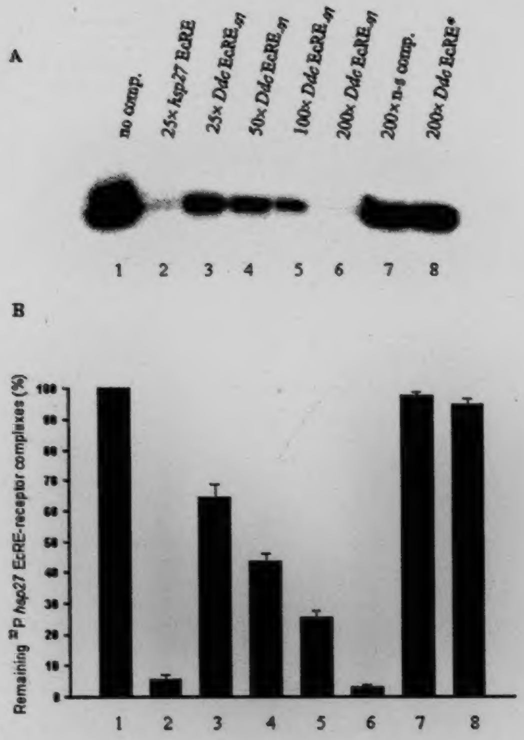

Figure 2.3. Relative affinities of *hsp27* and *Ddc* EcREs for the EcR/USP complex. A. Band shift of the ^{32}P-labelled *hsp27* EcRE probe by the EcR/USP heterodimer. Varying amounts of unlabeled oligonucleotides (see Fig. 2.1A for sequences) were added as competitors to assess the binding affinities. Non-specific competitor (n-s comp.) is a 30 bp oligonucleotide from the upstream *Ddc* region, which bears no similarity with the consensus EcRE. B. Quantitation of the amount of *hsp27* EcRE/receptor complex. The amount of radioactivity, detected in a gel slice from lanes 2-8 that included the complex, is expressed as a percentage of the bound probe, detected in a slice from the appropriate region of lane 1. The error bars show the range of values obtained in two experiments.

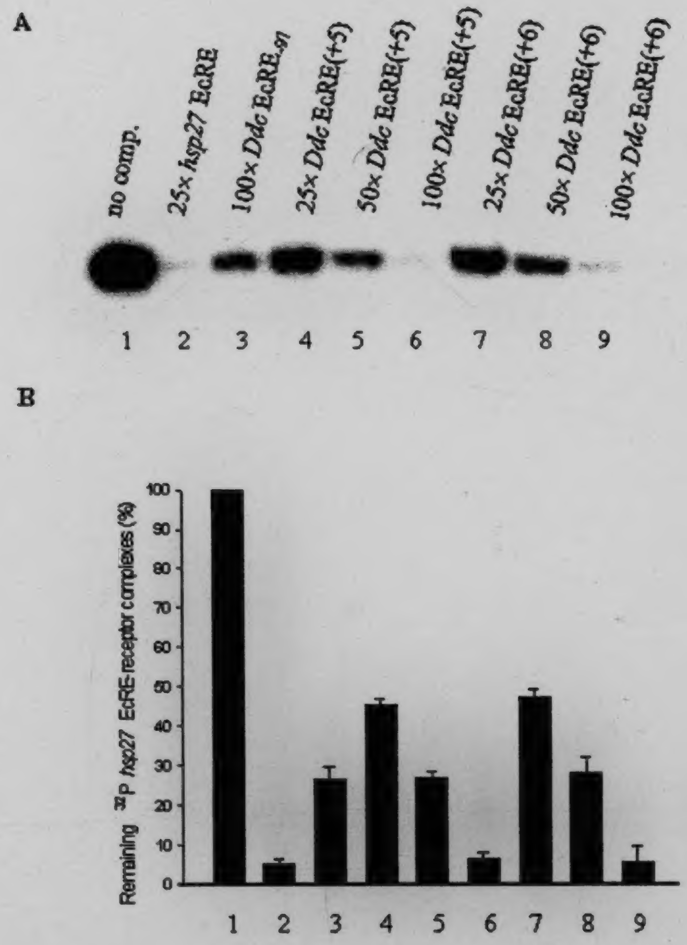

Figure 2.4. Relative affinities of wild-type and mutated *Ddc* EcREs for the EcR/USP complex. A. Binding of the EcR/USP complex to the ^{32}P-labelled *hsp27* EcRE probe in the presence of varying amounts of unlabeled competitor oligonucleotides (see Fig. 2.1A for sequences). B. The relative binding affinities in the reactions shown in lanes 2-9 (A.) were measured as described in the legend of Fig. 2.3. The error bars show the range of values in two experiments.

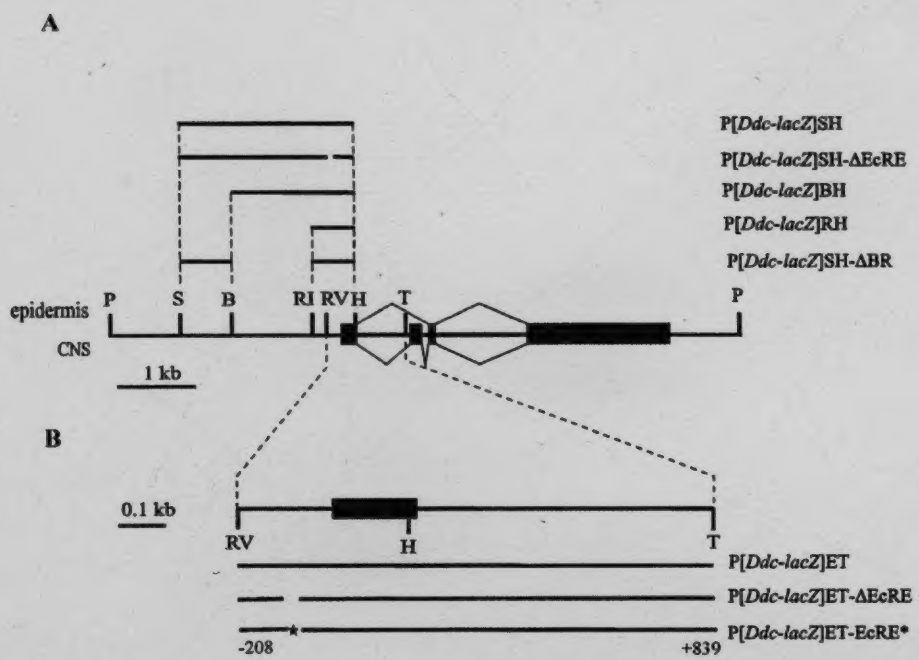
A
P[Ddc-lacZ]SH
P[Ddc-lacZ]SH-ΔEcRE
P[Ddc-lacZ]BH
P[Ddc-lacZ]RH
P[Ddc-lacZ]SH-ΔBR
epidermis
CNS
P
S
B
RI
RV
H
T
P
1 kb
B
0.1 kb
RV
H
T
P[Ddc-lacZ]ET
P[Ddc-lacZ]ET-ΔEcRE
P[Ddc-lacZ]ET-EcRE*
-208
+839

Figure 2.5. Schematic diagram of the *Ddc-lacZ* reporter constructs used in this paper. A. The neural- and epidermal-specific splicing patterns of the *Ddc* transcription unit are positioned on a restriction map of the 7.6 kb genomic fragment, which is sufficient for normal *Ddc* developmental expression. The black boxes represent exons. The sites shown are *Bsm*I (B), *Eco*RI (RI), *Eco*RV (RV*), Hpa*I (H), *Pst*I (P), *Sca*I (S) and *Taq*I (T). Reporter constructs extending upstream of the *Eco*RV site are shown above the map of the transcription unit. B. Reporter constructs extending downstream of the *Eco*RV site are shown on the enlargement of the *Eco*RV – *Taq*I fragment beneath the transcription map. The deletion of the presumptive EcRE element in P[*Ddc-lacZ*]ΔEcRE is indicated by a break in the line; the point mutations in P[*Ddc-lacZ*]ET-EcRE* are indicated by a star.

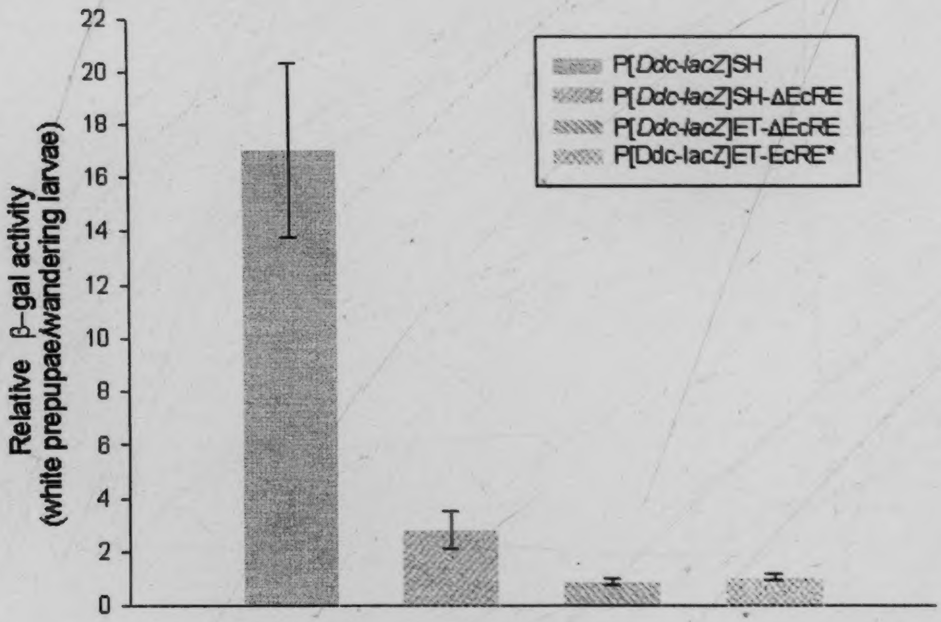

Figure 2.6. The effect of mutations in EcRE$_{-97}$. Reporter activity in white prepupae is expressed as a percentage of the activity of the early wandering third instar larvae. The value shown in the diagram is the average of two assays for at least three independent transgenic lines for each construct. The error bars indicate the standard deviations from the mean values.

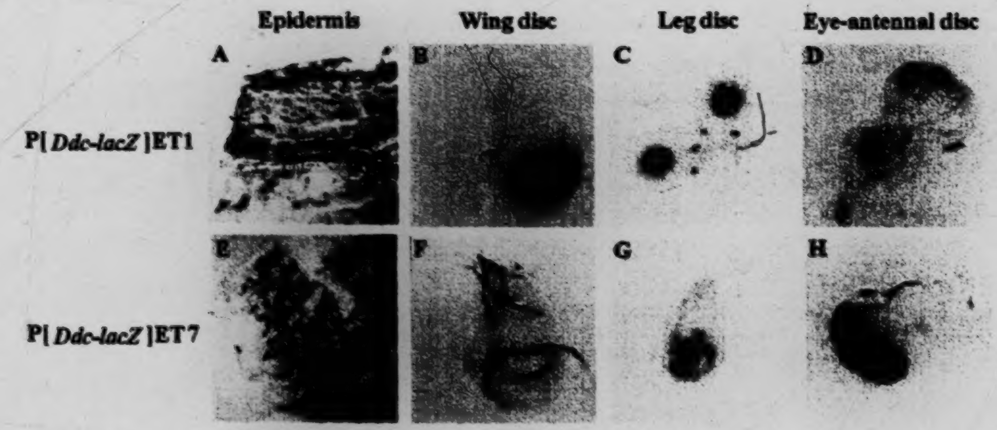
Epidermis
Wing disc
Leg disc
Eye-antennal disc
A
B
C
D
P[*Ddc-lacZ*]ET1
E
F
G
H
P[*Ddc-lacZ*]ET7

Figure 2.7. Epidermal expression patterns of two independent transgenic lines carrying P[*Ddc-lacZ*]ET reporter constructs extending downstream of –208 bp. Tissues were obtained from early wandering third instar larvae. The epidermis (panels A,E), wing (B,F,), leg (C,G) and eye-antennal (D,H) discs were stained for two hours to visualize β-galactosidase expression.

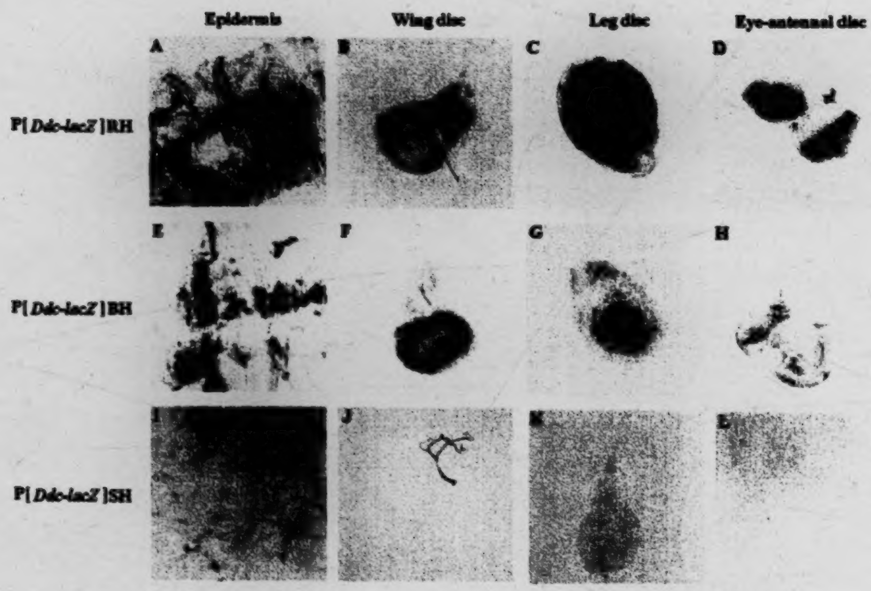
Epidermis
Wing disc
Leg disc
Eye-antennal disc
A
B
C
D
P[Ddc-lacZ]RH
E
F
G
H
P[Ddc-lacZ]BH
I
J
K
L
P[Ddc-lacZ]SH

Figure 2.8. Expression patterns in the epidermis and imaginal discs of transgenic lines carrying P[*Ddc-lacZ*] reporter constructs with various *Ddc* upstream regions. The *Ddc* fragments driving reporter expression in these lines share a common downstream end-point (at +174 bp) and extend to 382 bp (an *Eco*RI site), 1426 bp (a *Bsm*I site) or 2067 bp (a *Sca*I site) respectively (Fig. 2.1A). Tissues were dissected from early wandering larvae.

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Chapter 3

Control of dopa decarboxylase gene expression by the *Broad-Complex* during metamorphosis in Drosophila

Introduction

Drosophila metamorphosis is characterized by diverse developmental phenomena, including cell proliferation, tissue remodeling, cell migration, and programmed cell death. Cells undergo one or more of these processes in response to the steroid hormone 20-hydroxyecdysone (herein referred to as ecdysone) (Riddiford, 1993). Ecdysone first interacts with a heterodimeric nuclear receptor, composed of the EcR and USP subunits (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1992). Like other nuclear receptors, EcR/USP acts as a ligand-dependent transcription factor that modulates the activity of hormone-responsive target genes (Mangelsdorf *et al.*, 1995; Riddiford *et al.*, 2000). The hormone-receptor complex then activates a small set of ' early' polytene chromosome puffs. The products of these puffs are necessary, in turn, for the induction of a large number of 'late puffs' and the repression of the 'early' puffs themselves (Ashburner et al., 1974; Clever, 1964). Extensive genetic, cytogenetic and molecular analyses have shown that the *Broad-Complex* (*BR-C)*, which is located within the limits of the early ecdysone-induced puff at 2B3-5 on the X chromosome, is a critical mediator of the ecdysone hierarchy because it is required in the regulation of intermolt, early, and late gene activities (Belyaeva *et al.*, 1980; Karim *et al.*, 1993). Genetic functions carried by the *BR-C* gene are defined by mutations that comprise three groups of fully complementing alleles: *br* (*broad*), *rbp* (*reduced bristle on palpus*) and *2Bc* (Belyaeva *et al.*, 1980; Kiss *et al.*, 1988). Amorphic mutants lacking one of these functions die during metamorphosis at the late larval or early prepupal stages. Mutations that do not complement any of the other mutations define a group of *non-pupariating* (*npr*) alleles that correspond to a complete loss-of-function at the locus.

At the molecular level, approximately 100 kb of the genome is devoted to the *BR-C* locus (Bayer et al., 1996; DiBello et al., 1991). It consists of 11 exons, at least two transcriptional start sites and four polyadenylation sites. Alternative splicing of long primary transcripts gives rise to a family of four related proteins. All isoforms share a common amino-terminal core region but have a unique carboxy-terminus, which contains one of four pairs of zinc finger domains (Z1 to Z4). The first 120 residues of the core region defines a highly conserved BTB/POZ domain often found in developmentally regulated transcription factors (Zollman *et al.*, 1994). The BTB/POZ domain apparently mediates protein-protein interactions (Albagli et al., 1995; Bardwell and Treisman, 1994), which are involved in transcriptional regulation and chromatin remodeling (Dhordain *et al.*, 1997; Huynh and Bardwell, 1998). The complementation groups represent sub-functions corresponding to specific zinc-finger proteins: the *rbp*$^+$ function is carried by the Z1 isoform, the b*r*$^+$ function by the Z2 isoform and the *2Bc*$^+$ function by the Z3 isoform (Bayer et al., 1996; Bayer et al., 1997; Emery et al., 1994). Results of rescue experiments also support a partial functional redundancy between the different protein isoforms (Bayer *et al.*, 1997; Sandstrom *et al.*, 1997). The protein-genetic function assignment is, however, incomplete, because no specific function has as yet been determined for the Z4 isoform.

BR-C protein products are widely distributed among all tissues examined in the late larval to prepupal stages of development. All tissues studied to date contain all *BR-C* isoforms (Emery et al., 1994; Hodgetts et al., 1995; Huet et al., 1993). Their relative abundance, however, differs greatly from tissue to tissue, suggesting that the various members (or combinations) of the *BR-C* family of proteins function in different developmental pathways. For example, Z1 and Z3 are the predominant isoforms expressed in late third instar larval salivary glands, while at the same stage Z2 predominates in imaginal discs. The relative ratios of zinc-finger mRNA isoforms also change over time. Tested for induction in cultured imaginal discs, the Z2, Z3, and Z4 RNA isoforms accumulate to high levels at the beginning of the ecdysone response and abruptly disappear after several hours. In contrast, the Z1 RNA isoform continues to accumulate while the others decline, resulting in a switch in relative isoform levels. Thus, the expression of the four zinc-finger mRNA isoforms is dynamic, changing with time in a hormonally regulated sequence through the course of metamorphosis (Bayer *et al.*, 1996).

With the aim of further investigating the role of the *BR-C* protein isoforms as temporal regulators, we examined the requirement for *BR-C* in the expression of the *Dopa decarboxylase* gene (*Ddc*) as a model target gene. Control of *Ddc* during development involves several stage-specific regulatory hierarchies. Its expression in the epidermis at pupariation is that of a typical early-late ecdysone response gene (Ashburner and Richards, 1976), under the control of both the ecdysone-receptor and

BR-C (Chen et al., 2002; Clark et al., 1986; Hodgetts et al., 1995; O'Keefe et al., 1995). A mutation in the br^+ function substantially reduced the level of DDC in the epidermis of mature third instar larvae, from which a role for the Z2 isoform was inferred. Since a recombinant Z2 protein bound to five sites within the first intron of the *Ddc* gene, it was suggested that one or more of these sites was required for *Ddc* activity at pupariation (Hodgetts *et al.*, 1995). However, all four *BR-C* isoforms bound to the intron and many of the sites overlapped with one another. Thus, the functional significance of the intronic region was unresolved.

In this chapter, we examined the functional significance of the intronic BR-C binding sites in the regulation of epidermal *Ddc* expression. To our surprise, we found that these sites are not necessary for *Ddc* activity at pupariation; instead, they are indispensable for the activation of *Ddc* at eclosion. The sites upon which maximal *Ddc* expression at pupariation depends lie within the 5'-upstream region of *Ddc*. The emerging picture is that the *BR-C* is a critical regulator of target gene expression at several stages during metamorphosis. In addition to its synergistic interaction with the ecdysone receptor in the ecdysone-dependent events that occur at pupariation, our results show that the *BR-C* also plays a role at eclosion, a stage controlled, at least in part, by a non-ecdysteroid hormone axis (Baker et al., 1999).

Results

BR-C binding sites in the first intron are not necessary for epidermal Ddc expression at pupariation

Genetic experiments (Hodgetts et al., 1995; O'Keefe et al., 1995; Sandstrom et al., 1997) make it clear that *Ddc* expression in the larval epidermis at pupariation is under control of the Z2 transcription factor. Furthermore, a recombinant protein carrying the Z2 zinc finger was shown to bind to five sites within a *Dra*I-*Taq*I segment in the first intron (Hodgetts *et al.*, 1995). The *Dra*I-*Taq*I fragment is included in the P-element plasmid P[*Ddc-lacZ*]ET in which *lacZ* expression is under the control of the intact *Eco*RV-*Taq*I fragment (extending from –208 bp to +839 bp) of the *Ddc* gene (Fig. 3.1). This construct includes the functional ecdysone response element EcRE_{-97}, located between –97 bp and –83 bp, which is sufficient to confer a primary response on *Ddc* to ecdysone late in the wandering stage(Chen *et al.*, 2002). In order to test whether the BR-C binding sites in the first intron are functional *in vivo*, a quantitative assessment of the level of β-galactosidase in three transformed lines carrying P[*Ddc-lacZ*]ET was made in various *BR-C* mutant backgrounds. Three *BR-C* mutants were chosen for this assay; br^5 and the null $npr1^7$ alleles have DDC levels less than one third normal at pupariation whereas rbp^4 mutants exhibit enzyme levels equivalent to the wild type (Hodgetts *et al.*, 1995). The recessive lethal *BR-C* mutations were maintained as female heterozygotes in stocks carrying the X-chromosome balancer, *Binsn*. Male flies of the transgenic lines, which carry an insertion of the reporter construct on their third chromosome, were crossed to female flies from the appropriate *BR-C* mutant stock. Mutant and wild-type organisms were collected and assayed as described in the Materials and Methods. In presenting the results, *lacZ* activity of mutant males is expressed as a percentage of the activity of the wild-type sibs segregating in the cross. Two independent collections of organisms were assayed and the results are shown in Figure 3.2A. Reporter activities in P[*Ddc-lacZ*]ET-2 and P[*Ddc-lacZ*]ET-4 transformed lines were unaffected in any of the mutant hemizygotes. A slight decrease was caused by *npr1*^{7} in the P[*Ddc-lacZ*]ET-1 line, but the activity is still well above the level of 22% of normal DDC seen in *npr1*^{7} mutants (Hodgetts *et al.*, 1995). The absence of any mutant effects in *br*^{5} or *npr1*^{7}, the two alleles known to severely reduce DDC expression at pupariation, reveals that *BR-C* mediates its control of *Ddc* at this stage through a region not included in the first intron.

Deletions of the intronic BR-C binding sites result in no loss of reporter activity.

The unexpected results of reporter activity just presented predict that little effect on reporter expression in the epidermis at pupariation would be expected upon the removal of the *BR-C* binding sites from the *Eco*RV-*Taq*I fragment. To confirm this, two deletions were introduced into P[*Ddc-lacZ*]ET (Fig. 3.1). In the P[*Ddc-lacZ*]ΔInA construct, little other than the five Z2 binding sites was removed whereas P[*Ddc-lacZ*]ΔInB carries a larger intronic deletion. Seven transformed lines carrying P[*Ddc-lacZ*]ΔInA were recovered and the inserts were mapped unambiguously to a single chromosome. All these lines showed reporter expression in the epidermis of wandering third instar larvae as shown for P[*Ddc-lacZ*]ΔInA3 (Fig. 3.2B). Because a silencing element, located within the –2067 to –1427 bp region, is missing in P[*Ddc-lacZ*]ΔInA, the lines exhibited precocious *lacZ* expression, prior to wandering, in both the epidermis and in one or more of the major imaginal disc groups (leg, wing/haltere, eye/antennal) as we reported elsewhere (Chen *et al.*, 2002). The pattern of β-gal activity in transformants carrying P[*Ddc-lacZ*]ΔInA was not significantly different from that in pupariating larvae of P[*Ddc-lacZ*]ET-carrying lines (data not shown).

Eight lines carrying P[*Ddc-lacZ*]ΔInB were recovered and four were analyzed in some detail. Reporter activity in the larval epidermis was seen in all the lines as a typical preparation (for P[*Ddc-lacZ*]ΔInB1) shows (Fig. 3.2C). As with the lines carrying the smaller deletion, precocious reporter expression was seen in the epidermis and in a subset of the imaginal discs. The pattern of β-gal activity in pupariating larvae of transformants carrying P[*Ddc-lacZ*]ΔInB was indistinguishable from that in P[*Ddc-lacZ*]ET-carrying lines (data not shown). We conclude that the intronic *BR-C* binding sites are dispensable for up-regulating *Ddc* activity in epidermal tissues at pupariation.

Identification of a silencing element(s) whose loss results in premature reporter expression in pharate adults

Since the removal of the entire set of *Z2* binding sites had no effect on epidermal *Ddc* expression at pupariation, we examined whether these sites had any function in regulating *Ddc* expression at later developmental stages. DDC activity drops from the maximum at pupariation to a low basal level at about 60 hours after puparium formation (Kraminsky *et al.*, 1980). However, the wing (Fig. 3.3A), leg (Fig. 3.3B) and abdominal integument (data not shown) of transformed flies carrying P[*Ddc-lacZ*]ET all showed strong *lacZ* activity at this time. The precocious reporter activity seen in these lines leads to the conclusion that the endogenous *Ddc* gene is silenced in epidermal tissue during the pharate adult stage by an element(s) that lies outside of *EcoRV-TaqI* fragment. Since the larval silencing element located between –2067 and –1427 bp is missing in the P[*Ddc-lacZ*]ET construct, we tested whether transformants carrying P[*Ddc-lacZ*]BT (Fig. 3.1), which includes the upstream silencer, exhibited precocious reporter activity in pharate adults. The genomic region driving reporter expression in this construct extends downstream from a *Bgl*II site located at –2.7 kb to the *Taq*I site within the first intron (Fig. 3.1). Although transformants carrying this construct showed no precocious β-galactosidase activity in the larval epidermis (data not shown) nor in the imaginal discs (Fig. 3.4C and D), they exhibited very strong hypodermal staining in pharate adults (Fig. 3.3E, F). Since the *PstI* genomic fragment (Fig. 3.1) can specify the entire temporal and spatial pattern of *Ddc* activity during development (Scholnick *et al.*, 1983), the missing pharate adult stage-specific silencer is most likely located within the second intron or 3'UTR of *Ddc*. In its absence, reporter expression appears in the hypoderm about 30 hours after pupariation, possibly activated by the large surge of ecdysone that occurs about this time (Kraminsky *et al.*, 1980).

Deletion of BR-C binding sites in the first intron results in loss of Ddc activity at eclosion

We observed a striking difference in *Ddc*-driven reporter expression at adult eclosion between lines carrying the intact *EcoR*V-*Taq*I fragment and lines carrying the deletion that specifically removes all the *BR-C* sites (Fig. 3.4). Both P[*Ddc-lacZ*]ET lines and P[*Ddc-lacZ*]ΔInA lines showed considerable precocious β-galactosidase activities in pharate adults (PA) due to the absence of the silencer described above. Nevertheless, β-galactosidase levels in the three P[*Ddc-lacZ*]ET lines increased almost three-fold at eclosion (NE) while enzyme levels in the three P[Ddc-lacZ]ΔInA transformants declined from their pre-eclosion level. The rapid decrease in β-galactosidase activity in P[*Ddc-lacZ*]ET transformants following eclosion reflects a similar drop in activity of the endogenous *Ddc* gene at this stage (Kraminsky *et al.*, 1980). The differences in β-galactosidase activity between transformants carrying the two constructs disappeared between 48 and 120 hr post-eclosion.

All BR-C RNA isoforms are present at eclosion

The above data show that the 217 bp *Ddc* intronic region, containing the BR-C binding sites, is required to induce reporter expression in a manner that mimics the induction of the endogenous *Ddc* gene. To test the prediction that one or more of the *BR-C* RNA isoforms is present at this stage, a reverse transcriptase-polymerase chain reaction (RT-PCR) approach was used. Total RNA was isolated from whole organisms collected as white prepupae or as adults, within 1 hour of eclosion. Isoform specific primers (Hodgetts *et al.*, 1995) revealed that all four of the zinc finger classes could be detected at both stages (Fig. 3.5). Differences may exist in the relative amounts of each isoform at the two stages but no attempt was made to make this analysis strictly quantitative.

Identification of an upstream BR-C interacting region

In order to define the region through which the *BR-C* mediates up-regulation of *Ddc* in the epidermis of pupariating larvae, we crossed three sets of transformed lines carrying variously deleted *Ddc* 5'-upstream regions into *BR-C* mutant backgrounds and analyzed β-galactosidase activity (Fig. 3.6). All these reporter constructs shared a common downstream endpoint at a +174 *Hpa*I site. Reporter activity in line P[*Ddc-lacZ*]RH, in which *lacZ* was driven by the *Eco*RI-*Hpa*I fragment, was unaffected by any of the three *BR-C* mutant alleles tested. This indicates that the *cis*-acting region lies upstream of the *Eco*R1 site at –383 bp. By contrast, transformants carrying an upstream region that extended to the *Bsm*I site at –1426 bp showed definite allele-specific mutant effects on β-galactosidase activity. In both br^5 and $npr1^7$ mutant organisms, β-galactosidase activity dropped to about one third of the activity in rbp^4 mutants. Likewise, a transformant carrying the construct with an upstream endpoint at the –2067 bp *Sca*I site showed reduced activity in br^5 and $npr1^7$ mutants. These results confirm that the *BR-C* acts through a region between *Bsm*I and *Eco*RI (–1426 bp to –383 bp) to up-regulate *Ddc* expression.

BsmI-EcoRI contains Z2 isoform binding sites

The 1044 bp *Bsm*I-*Eco*RI fragment was subcloned into three contiguous sub-fragments and subjected to mobility shift experiments with the recombinant protein isoforms, *BR*-C$_{Z1-Z3}$, produced by overexpression in *E. coli* and purified to 90% homogenity (Dubrovsky et al., 2001). A 360 bp sub-fragment within the *BsmI-EcoRI* region (-383 to –743 bp) showed extensive retardation by Z2 (Fig. 3.7) and the binding proceeded through one intermediate at non-saturating level of protein (lane 2). The appearance of the intermediate can be explained by the existence of multiple binding sites within the fragment (see below). The binding to this 360 bp probe was eliminated in the presence of a 50-fold excess of unlabelled fragment (data not shown) and additional binding studies revealed little Z2 binding occurred to the two other sub-fragments within the *Bsm*I-*Eco*RI region. Unlike the intronic BR-C binding region, within which tightly clustered binding sites of all four isoforms exist (Hodgetts et al., 1995), the 360 bp probe exhibited no retardation with Z1or Z3 (data not shown).

The 360 bp fragment was subjected to DNase I footprinting using the recombinant Z2 protein (Fig. 3.8). Three obvious footprints were resolved on the coding strand (Fig. 3.8A). These footprints were numbered beginning at the promoter proximal site. The extent of each footprint is shown beside the autoradiograph. Analysis of the non-coding strand (Fig. 3.8B) confirmed the existence of these three footprints. Within site 1, Z2 binding produced a band of DNase I cleavage in the middle (see corresponding arrowhead in Fig. 3.8A). At site 3, Z2 binding protected a more extended area. When the sequences within the three footprints were aligned

(Fig. 3.9), footprint 3 revealed two binding sites, consisting of direct repeats separated by eight nucleotides. In previous studies of *Sgs-4* (von Kalm et al., 1994), *L71-6* (Crossgrove et al., 1996), *hsp23* (Dubrovsky et al., 2001), and the *Ddc* intronic BR-C binding sites (Hodgetts et al., 1995), the BR-C isoforms bound a variable 9-12 nucleotide sequence that contained a conserved central core element. Here, we observed a similar pattern, with the central core element being a CTA triplet, perfectly conserved at all of the Z2 binding sites reported to date (Fig. 3.9).

In order to confirm the functional importance of these Z2 binding sites, two deletions were introduced into the P[*Ddc-lacZ*]SH construct. P[*Ddc-lacZ*]SH-ΔBsR carried a deletion that removed the entire *BsmI-EcoRI* fragment; P[*Ddc-lacZ*]SH-ΔZ2 removed only the 360 bp sub-fragment that was used in the footprinting analysis. Transformed lines carrying these deletion constructs were crossed into *BR-C* mutant backgrounds and β-galactosidase activity was measured. None of the transformed lines exhibited the allele-specific mutant effect manifested by P[*Ddc-lacZ*]SH-carrying lines (data not shown). The failure to observe reduced reporter expression in the mutant genetic background indicates that the *cis*-acting region through which *BR-C* acts is missing from the *Ddc* genomic region driving *lacZ* expression in both these constructs.

EcRE$_{-97}$ *and BR-C binding sites are necessary to mediate the induction of Ddc*

Between the early wandering stage and pupariation, reporter activity in P[*Ddc-lacZ*]SH carrying transformed flies increased on average 16-fold (Fig. 3.10). This is comparable to the endogenous *Ddc* induction at this stage. Recently we have identified an ecdysone response element, EcRE$_{-97}$, just upstream of the *Ddc* transcription start site that is required for full *Ddc* induction (Chen et al., 2002). In order to determine whether the ecdysone-receptor complex and BR-C are both necessary to mediate the full induction, we constructed reporters (see Fig. 3.1), which eliminated EcRE$_{-97}$ (P[*Ddc-lacZ*]SH-ΔEcRE), or the upstream BR-C binding sites (P[*Ddc-lacZ*]SH-ΔZ2), or both (P[*Ddc-lacZ*]SH-DΔ). A deletion of either EcRE$_{-97}$ or the Z2 binding sites resulted in about a 3-fold induction during wandering (Fig. 3.10), demonstrating the necessity of these regulatory elements. Since the double deletion totally abolished the induction at pupariation (Fig. 3.10), the two *cis*-acting elements are both necessary to mediate a full response. Each of the two essential regulatory sequences contributed a 3-fold induction. If the individual responses were additive, a 6-fold induction would have been expected. Since the intact regulatory region produced a 16-fold increase, the data strongly suggest that a synergistic interaction occurs between the ecdysone-receptor and the BR-C protein.

Z2 may physically interact with the ecdysone receptor in vitro

The *Ddc* ecdysone response element, EcRE$_{-97}$, exhibits a four- to eight-fold lower affinity *in vitro* than that of the canonical *hsp27* EcRE for the receptor complex (Chen et al., 2002). The synergy between the ecdysone receptor and Z2 seen in late third instar (Fig. 3.10), implies that a direct interaction between Z2 and ecdysone receptor might enhance the affinity of ecdysone receptor complex for EcRE$_{-97}$. To explore this possibility, we used a gel mobility assay. Z2 protein, in the presence or absence of the 360 bp *Ddc* fragment to which it binds, was mixed with the ecdysone receptor, EcR/USP, and then the ability of the latter to form a specific complex with labeled EcRE$_{-97}$ was tested. Addition of just the lysate, produced retardation of the probe by an unknown protein in the lysate (lane 2, Fig. 3.11A, arrowhead). Addition of just the receptor produced a specific retarded band (lane 3, Fig. 3.11A; arrow) not seen in the control lanes (1, 2). Z2 on its own was unable to bind to EcRE$_{-97}$ (lane 4, Fig. 3.11A) or influence the formation of the complex formed with the unknown reticulocyte lysate protein (lane 5). However, when Z2 and the EcR/USP complex were mixed together in the absence of the EcRE$_{-97}$ probe, the affinity of ecdysone receptor complex for the EcRE$_{-97}$ probe was greatly reduced (compare lane 3 with lanes 7 and 8 in Fig. 3.11A). There seems to a slight reduction in the amount of the receptor/EcRE complex caused by the 360 bp fragment on its own (lane 3 versus 6 in Fig. 3.11A). This accounts for the lower amount of receptor/EcRE complex in lane 8 compared to lane 7. Although this result suggests that Z2 and the ecdysone receptor interact, it was contrary to our expectations of enhanced receptor binding in the presence of Z2. However, it should be noted that in late third instar larvae, *BR-C* is induced by the ecdysone receptor. Therefore, the EcRE$_{-97}$ would be occupied by the receptor before BR-C proteins had begun to appear. To test whether Z2 could remove the receptor after it had bound to EcRE$_{-97}$, the experiments in Fig. 3.11B were carried out. In fact, Z2 was unable to alter the amount of the receptor/EcRE complex, if the receptor was allowed to bind to the EcRE$_{-97}$ probe for a short period of time before addition of the Z2 protein (compare lane 1 with lane 3 or lane 2 with lane 4).

Discussion

A mutation in the *BR-C br* function substantially reduces the level of DDC in the epidermis of mature third instar larvae (Hodgetts *et al.*, 1995). Since the br^{28} allele is caused by a transposon insertion into the Z2 DNA binding domain (DiBello *et al.*, 1991), we concluded that a product of the *BR-C* locus carrying this motif mediates the rapid appearance of DDC in mature larvae in response to an elevated titer of ecdysone. Both the transcript and a protein carrying the Z2 DNA-binding domain are present in the epidermis, and a *BR-C* recombinant protein carrying the Z2 finger binds to five sites within the first intron of the *Ddc* gene (Hodgetts *et al.*, 1995). In this paper we tested whether these *BR-C* binding sites mediate Z2 action *in vivo* and to our surprise, found that all five sites are dispensable for *Ddc* induction at pupariation. Instead, deletion of these sites resulted in loss of induction at eclosion, suggesting that these sites play an important role in mediating *BR-C* function at this stage.

Despite the clear dependence of *Ddc* expression on both a high ecdysone titer and *BR-C* function in the epidermis of late third instar larvae, no such dependence is apparent in the appearance of DDC at the first three molts. At the molt from second to third instar, the increase in DDC activity is associated with a declining ecdysone titer and was shown to be independent of *BR-C* (Hodgetts *et al.*, 1995). Likewise, *BR-C* independence of *Ddc* induction at the two prior molts can be inferred, since none of the *BR-C* mutants die at these molts. Eclosion resembles the first three molts during development in that the peak of DDC activity occurs after the ecdysone titer has dropped from a maximum attained earlier (Kraminsky *et al.*, 1980). However, the loss of DDC activity at eclosion, due to deletion of the *BR-C* binding sites in the first intron, indicates that the *BR-C* is required for *Ddc* expression at this stage. The low titer of ecdysone at eclosion makes it very unlikely that the *BR-C* participates in the kind of co-operative interaction with the ecdysone receptor that we envision at pupariation (Chen *et al.*, 2002). In fact, the *BR-C* transcription itself could occur in an ecdysone-independent fashion at eclosion. The developmental cues that control eclosion have not been studied in much detail. However, it appears that an entirely different hormonal regime from that which directs the onset of metamorphosis operates. It is based on the eclosion hormone (EH) whose presence is required for eclosion in *Drosophila* (Baker *et al.*, 1999). Immunocytochemical studies show that EH is released prior to ecdysis of the third instar larva and prior to adult eclosion, suggesting a function during at least these two ecdyses (Horodyski *et al.*, 1993). In pharate adults, the final release of EH normally occurs approximately 40 min before ecdysis. How it might control the *BR-C*-dependent epidermal gene cascade in which *Ddc* is a target remains a subject for further investigation.

All four *BR-C* isoform transcripts can be detected at eclosion (Fig. 3.5). Since every tissue tested to date contains all four of the major *BR-C* isoform classes (Emery et al., 1994; Hodgetts et al., 1995; von Kalm et al., 1994), and the deleted *Dra*I-*Taq*I region in the first intron contains binding sites for all four of the *BR-C* recombinant proteins (Hodgetts *et al.*, 1995), it is possible that a *BR-C* isoform other than Z2 is responsible for the *Ddc* activation at eclosion. In cultured imaginal discs, there is an isoform switch from accumulations of Z2, Z3 and Z4 to the Z1 isoform, 6 hours after incubating discs with ecdysone (Bayer *et al.*, 1996). This switch is also manifested at puparium formation, where a dramatic shift from Z2 to Z1 occurs (Emery *et al.*, 1994). The precipitous nature of the switch in zinc finger isoform expression suggests that different isoforms have different functions in the temporal regulation of downstream target genes. All four isoforms are present at eclosion (Fig. 3.5), thus opens up the possibility that a different isoform other than Z2 mediates DDC activity during eclosion.

Recombinant *BR-C* protein isoforms are known to bind to several target genes in Drosophila, including *L71-6,* (Crossgrove et al., 1996), *hsp23,* (Dubrovsky et al., 2001), *Sgs-4* (von Kalm et al., 1994) and *rpr* (Jiang et al., 2000). Furthermore, antibodies directed against the core domain of the BR-C proteins bind to more than 300 sites on salivary gland polytene chromosomes (Gonzy et al., 2002), indicating a large battery of target genes exists for the proteins encoded by the locus. However, care must be exercised in the interpretation of *in vitro* BR-C binding. In *L71-6*, *hsp23* and *Sgs-4,* mutation or deletion of the BR-C isoform binding sites eliminated normal reporter gene activity. However, in the case of *Sgs-4* where *BR-C* is required for normal activation in mid-third instar larvae (von Kalm et al., 1994), the mutations also altered Fkh binding sites that are required for up-regulation of *Sgs-4* expression. Therefore, the loss of reporter expression in the mutants may be unrelated to BR-C binding of these sites. Since BR-C proteins are present at 98D, the cytogenetic region that includes *fkh*, it has been suggested that *BR-C* controls *Sgs-4* indirectly via its control of *fkh* (Renault et al., 2001). In the case of *rpr*, *BR-C* is known to exert its effects through a 1.3 kb promoter region. However, deletion of the Z1 binding sites within this fragment had no effect on reporter activity, leading the authors to conclude that another *BR-C*-dependent transcription factor was probably acting within the 1.3 kb genomic region (Jiang et al., 2000).

Since the deletions of the *in vitro* BR-C binding sites encompassed little more than the domain of the BR-C binding sites themselves, the up-regulation of *Ddc* activity is likely a direct action of *BR-C* on the *Ddc* regulatory regions. The *cis*-acting element through which *BR-C* regulates *Ddc* expression at pupariation was mapped between –743 and –383 bp. The four Z2 recombinant protein binding sites, located in this region, shared a perfect conserved triplet core sequence among themselves and with those present at other *BR-C* regulated genes. Consensus DNA-binding sequences have been defined for each of the four isoform-specific zinc-finger domains. Despite the many possible binding sites that exhibit the consensus core sequences within the 360 bp region between –743 and –383 bp, no binding of the *BR-C* protein isoforms other than Z2 was detected. The variability of these A/T-rich sequences suggests that along with the zinc-finger domain, additional determinants on the protein are important for specific DNA recognition. One such determinant could be the BTB/POZ domain that is present in all BR-C isoforms. The crystal structure of the BTB domain of PLZF (promyelocytic leukemia zinc finger) was determined and revealed to form a homodimer (Ahmad et al., 1998). The domain mediates a functionally relevant dimerization *in vivo*, promoting strong cooperative DNA binding to multiple sites (Bardwell and Treisman, 1994). BTB/POZ domains are thus likely to play a critical role in targeting proteins to specific sites. The synergy between the ecdysone-receptor and Z2 seen in late third instar (Fig. 3.10), suggested that a direct interplay between these two transcription factors controls *Ddc* expression. In fact, when the two proteins were mixed in the absence of EcRE$_{-97}$, Z2 reduced the affinity of the receptor for its target. However, BR-C was unable to interact with the receptor once it had bound to EcRE, a situation that more closely resembles what occurs *in vivo*. Thus, the synergy we detect could result from independent interactions of BR-C and the receptor with the basal transcription complex.

The finding that the *Ddc* gene is actively repressed in pharate adults by what we call the downstream silencer, was an unexpected finding in this work. It is however, quite reminiscent of our discovery of an upstream *cis*-acting sequence that silences *Ddc* prior to pupariation (Chen et al., 2002). The rise in titer of ecdysone, which begins 18-19 hours after puparium formation (APF), results in the apolysis of the pupal cuticle. In the absence of the downstream silencer, *Ddc* activity would appear prematurely in the epidermal tissue, as evidenced from transformants carrying P[*Ddc-lacZ*]BT, which start to show *lacZ* staining at this time (data not shown). Formation of the adult cuticle commences well after the apolysis of the pupal cuticle and after the cellular morphogenesis that produces bristles and hairs (Mitchell et al., 1983; Reed et al., 1975; Roter et al., 1985). The onset of adult cuticulin deposition occurs 15-25 hours after the peak in hormone titer. Moreover, even when cuticulin deposition begins, it is protracted over a 10-15-hour period. This deliberate pace of cuticulin deposition provides additional time for morphogenesis. Deposition of most procuticle components occurs between 55 and 80 hours APF when the titer of ecdysone is falling or is low. Thus, repression of *Ddc* in the epidermis insures that tanning and hardening of the adult cuticle does not occur until after this extended period of adult cuticle deposition and maturation. Release of *Ddc* from the repressed state might be triggered by the falling ecdysone titer, as DDC activity starts to rise 80 hours APF.

The identity of the repressor that acts through the downstream silencer remains to be worked out. Genetic and transgenic analysis of *BR-C* regulation of downstream genes shows that different *BR-C* isoforms can have opposite regulatory effects on the same target gene. In one case, the Z3 isoform activates the larval fat body-specific expression of the *Fbp-1* gene and the Z2 protein represses it (Mugat *et al.*, 2000). In another case, Z3 acts as a repressive isoform for the expression of a salivary gland specific gene *Sgs4*, while Z1 activates it (Bayer *et al.*, 1996). Thus, the switch from one predominant isoform to another may mediate the precise temporal control of target genes. Although the sensitivity of the Northern analysis used to detect *BR-C* expression needs to be addressed, we feel that the absence of *BR-C* mRNA during the pharate adult stage (Zhou and Riddiford, 2002), makes it unlikely that an isoform of *BR-C* functions as a repressor during this stage.

Materials and methods

Drosophila crosses

Stocks used in this study were maintained at 25°C on a standard cornmeal/molasses medium. *BR-C* mutant chromosomes marked with *yellow (y)* were carried in females balanced over *Binsn*, an X-chromosome carrying the markers

Bar and *singed*. Male flies of transgenic lines carrying autosomal P-element insertions were crossed to *BR-C* mutant female flies. Male larvae were collected as white prepupae. Mutant larvae were distinguished from their wild-type sibs by the reduced pigmentation of their denticle belts and mouth parts. Developmental arrest of the *br*^{5} mutants occurred at puparium formation and the organisms failed to tan the larval cuticle or evert their imaginal discs. The *rbp* ^{4} mutants arrested development somewhat later in the pupal or pharate adult stages. The *npr1*^{7} animals never pupariated and eventually died after several days as wandering third instar larvae; in this case the males were collected as immobile third instar organisms.

Plasmid construction

The extent of the *Ddc* region driving *lacZ* expression in the *lacZ* reporter plasmids used in this study is shown in Fig. 3.1. The assembly of P[*Ddc-lacZ*]ET, P[*Ddc-lacZ*]SH, P[*Ddc-lacZ*]BsH, and P[*Ddc-lacZ*]RH has been described elsewhere (Chen *et al.*, 2002). P[*Ddc-lacZ*]BT, P[*Ddc-lacZ*]ΔInA, and P[*Ddc-lacZ*]ΔInB were derivatives of P[CaSpeR-AUG-β-gal], which carries a *lacZ* reporter gene in a translational fusion with a fragment from the *Adh* gene that excludes the promoter and transcription initiation site (Thummel *et al.*, 1988). Into this vector we cloned fragments from the *Ddc* genomic region that included its transcription start site. P[*Ddc-lacZ*]BT extended downstream from the *Bgl*II site located at –2.7 kb to a *Taq*I site within the first intron. The *Bgl*II-*Taq*I fragment was amplified using two primers: 5'-GGGATCCAGGTCATCA-GATCTCATCGTAT-3' (forward) and 5'-CGGGATCCTCGAATGGTTAGAGCTAATATG-3' (reverse). *Bam*HI recognition sites (underlined) were included in the primers to facilitate subsequent cloning. The product amplified by *Pfu* (Stratagene) was inserted into the *Eco*RV site of pBluescript and subsequently subcloned into P[CaSpeR-AUG-*β*-gal] as a *Bam*HI fragment. The desired orientation was confirmed by PCR. P[*Ddc-lacZ*]ΔInA and ΔInB carried different deletions of the *BR-C* binding sites within the first *Ddc* intron. These were introduced into the *Eco*RV-*Taq*I fragment after it had been modified to include two unique restriction sites: a *Kpn*I site at position +586 bp followed by a *Not*I site, 6 bases further downstream. These restriction sites were introduced by *in vitro* mutagenesis of the *Ddc* genomic sequence using Muta-Gene (Bio-Rad) and facilitated the introduction of the deletions, using Erase-A-Base (Promega). The constructs were sequenced to ascertain their exact end-points; plasmid P[*Ddc-lacZ*]ΔInA carried a deletion between +581 and +798 bp and plasmid P[*Ddc-lacZ*]ΔInB carried a deletion between +268 and +839 bp.

All other plasmids used in this paper were assembled from pPelican, a derivative of p[CaSpeR-AUG-*β*–gal] that carries a *gypsy* insulator on either side of the *lacZ* reporter gene (Barolo *et al.*, 2000). The reporter plasmid carrying the *Bsm*I–*Eco*RI deletion within the *Sca*I–*Hpa*I fragment (P[*Ddc*–*lacZ*]SH-ΔBsR) was created by inverse PCR using the *Sca*I–*Hpa*I insert in pBluescript SK(+) (Chen *et al.*, 2002) as the template and primers: 5'-AAGTATTTCTCCGCTTATAGG-3' (-1446 to –1427 bp) and 5'-GGAAGCACGTGAGCAGAAT-3' (-379 to –398 bp). The desired fragment was released from this plasmid as a *Pst*I-*Kpn*I fragment and subcloned into pPelican. The P[*Ddc*–*lacZ*]SH-ΔZ2 construct was made in similar way by inverse PCR amplification of the *Sca*I–*Hpa*I insert in pBluescript SK(+) using the primer pair:

5'-GGCCGGAATTTCAAAATTCA-3' (–813 bp to -794) and 5'-GGAAGCACGTGAGCAGAAT-3' (-379 to –398 bp).

To make the ΔEcRE deletion, the *Eco*RV-*Nco*I fragment from the *Sca*I-*Hpa*I insert in pBluescript SK(+) was replaced with the *Eco*RV-*Nco*I fragment from P[*Ddc-lacZ*]ET-ΔEcRE (Chen *et al.*, 2002). The resulting SH-ΔEcRE in pBluescript was released as a *Pst*I-*Kpn*I fragment and subcloned into pPelican. P[*Ddc-lacZ*]SH-DΔ was made by inverse PCR amplification of pBluescript SH-ΔEcRE using the same primer pair as to make P[*Ddc-lacZ*]SH-ΔZ2. Again, the resulting SH-DΔ in pBluescript was released as a *Pst*I-*Kpn*I fragment and subcloned into pPelican.

RT-PCR

RT-PCR to detect *BR-C* transcript levels in the epidermis was carried out as described elsewhere (O'Keefe *et al.*, 1995). RNA was obtained for amplification from whole organisms. The RNA (1 μg) was primed with oligo(dT) and transcribed by reverse transcriptase (Invitrogen). The subsequent DNA amplification was carried out on 1/5 of the cDNA using the PCR buffer, Q-solution and Taq polymerase from Qiagen. The *BR-C* primers (Hodgetts et al., 1995) were used except for Z1: 5'-CTGCTTGAGGTTGCGGTGGTAG-3'. chosen to amplify each of the zinc-finger-binding domains. All four of the PCR reactions were anchored with a common primer (C) located at the 3' end of the core region of *BR*-C. The other primers were located just within or immediately 3' to the respective zinc finger motifs. The reactions were carried out as follows: cycle 1: 94°C for 3 min, 58°C for 45 sec and

72°C for 2 minutes followed by 28 repetitions of cycle 1 except that the denaturation step was 92°C for 45 sec. After 3 cycles, the program was interrupted while primers for the *rp49* control were added: 5'-AGTAAACGCGGGTTCTGC-AT-3' (forward) and 5'-AGCATACAGGCCCAAGATCG-3' (reverse).

Gel mobility shift assays

Mobility shift assays using the EcRE$_{-97}$ probe were carried out as before (Chen et al., 2002). Receptor protein was generated *in vitro* from the expression vectors CMX-EcR-B1 and CMX-USP (Yao et al., 1992) that were used as templates for coupled transcription/translation in a rabbit reticulocyte lysate (Promega). The translated proteins were incubated in binding buffer, which contained 100 mM KCl, 7.5% glycerol, 20 mM HEPES (pH 7.5), 2 mM dithiothreitol and 0.1% NP-40, on ice for 20 min in the presence of 2 µg of nonspecific competitor poly(dI-dC) and 100 ng of Z2 protein (+/- the 360 bp sub-fragment DNA as indicated in the figure legend). Approximately 1 ng of the ^{32}P end-labeled EcRE$_{-97}$ probe (10^8 cpm/µg) was added to the receptor protein mixture and the binding reaction was allowed to proceed at room temperature for 20 minutes. Alternatively, the ecdysone receptor was incubated with the EcRE$_{-97}$ probe on ice for 20 minutes before the addition of the Z2 protein (+/- the 360 bp subfragment).

Recombinant *BR-C* proteins (von Kalm *et al.*, 1994) were used in mobility shift assays as described elsewhere (Hodgetts *et al.*, 1995). *Ddc* DNA fragments from the regions of interest were subcloned into pGemT-EASY (Promega) through PCR amplification using primers that contained an *Ava*I site at the 5' end. The *Ava*I fragment was released from the vector by digestion and purified from a 1.2% agarose gel, labeled by end-filling the restriction sites using Klenow and purified using a QIAEX II kit (Qiagen). Binding was carried out for 60 minutes on ice with 4-6 x 10^4 cpm of the labeled probe, 2μg of non-specific competitor poly(dI-dC), and up to 200 ng of *BR-C* protein in a 20 μl reaction volume containing 50 mM KCl, 10% glycerol, and 20 mM Tris-HCl (pH 7.5). The reaction was then loaded on a 4% non-denaturing polyacrylamide gel in a 0.5X TBE running buffer. After electrophoresis, the gel was dried for autoradiography.

DNase I footprinting analysis

Recombinant *BR-C* Z2 protein was produced in *E. coli* and purified as described in von Kalm et al. (1994). A 360 bp *Ddc* DNA fragment that covered the region of interest was amplified with primers that contained *Ava*I sites at either end and subcloned into pGemT-EASY (Promega). The PCR fragment was released with *Ava*I and *Sac*II digestion, labeled by end-filling the *Ava*I site using Klenow and purified using QIAEX II (Qiagen). DNase I footprinting analysis was performed as described (Dubrovsky *et al.*, 2001). Purified protein (100-400 ng) was incubated with the labeled DNA fragment (4-6 ng) for 30 min at room temperature in 50 μl of 25 mM HEPES, pH 7.9, 50 mM KCl, 6 mM $MgCl_2$, 0.05 mM EDTA, 0.5 mM DTT, 10 μM $ZnSO_4$, 2% PVA, 5% glycerol, and 10 μg/ml of poly (dI-dC). After incubation, 50 μl of DNase I (2 μg/ml in 5 mM $CaCl_2$, 10mM $MgCl_2$) was added and digestion was allowed to proceed for 2 min at room temperature. The reaction was terminated with an equal volume of stop solution (0.2 M NaCl, 1% SDS, 20 mM EDTA, 250 μg/ml tRNA). DNA samples were precipitated with ethanol, fractionated on a standard sequencing gel and visualized by autoradiography.

Germline transformations and histochemical assays

Injections and staining of the integument and the imaginal discs for *lacZ* activity and the measurement of β-galactosidase activity on crude extracts were carried out as described elsewhere (Chen *et al.*, 2002).

Acknowledgments

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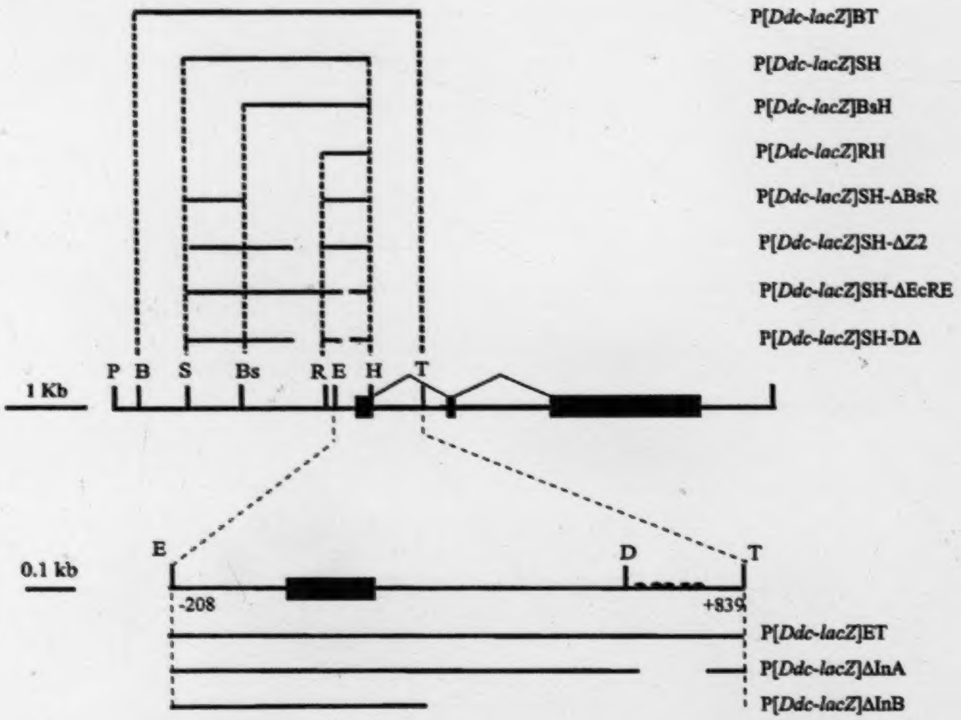
P[*Ddc-lacZ*]BT
P[*Ddc-lacZ*]SH
P[*Ddc-lacZ*]BsH
P[*Ddc-lacZ*]RH
P[*Ddc-lacZ*]SH-ΔBsR
P[*Ddc-lacZ*]SH-ΔZ2
P[*Ddc-lacZ*]SH-ΔEcRE
P[*Ddc-lacZ*]SH-DΔ
1 Kb
P
B
S
Bs
R
E
H
T
0.1 kb
E
D
T
-208
+839
P[*Ddc-lacZ*]ET
P[*Ddc-lacZ*]ΔInA
P[*Ddc-lacZ*]ΔInB

Figure 3.1. Schematic diagram of the P[*Ddc-lacZ*] reporter constructs used in this paper. The epidermal-specific splicing pattern of the *Ddc* transcription unit is positioned on a restriction map of the 7.6 Kbp *Pst*I-*Pst*I genomic fragment, which is sufficient for normal *Ddc* developmental expression. The sites shown are *Bgl*II (B), *Bsm*I (Bs), *Dra*I (D), *Eco*RI (R), *Eco*RV (E), *Hap*I (H), *Pst*I (P), *Sca*I (S), and *Taq*I (T). Reporter constructs whose 5' ends located upstream of the *Eco*RV site are shown above the map of the transcription unit; reporter constructs extending downstream from the *Eco*RV site are shown on the enlargement of the *Eco*RV – *Taq*I fragment beneath the transcription map. The five Z2 binding sites previously mapped within the first intron (Hodgetts et al., 1995) are shown above the line.

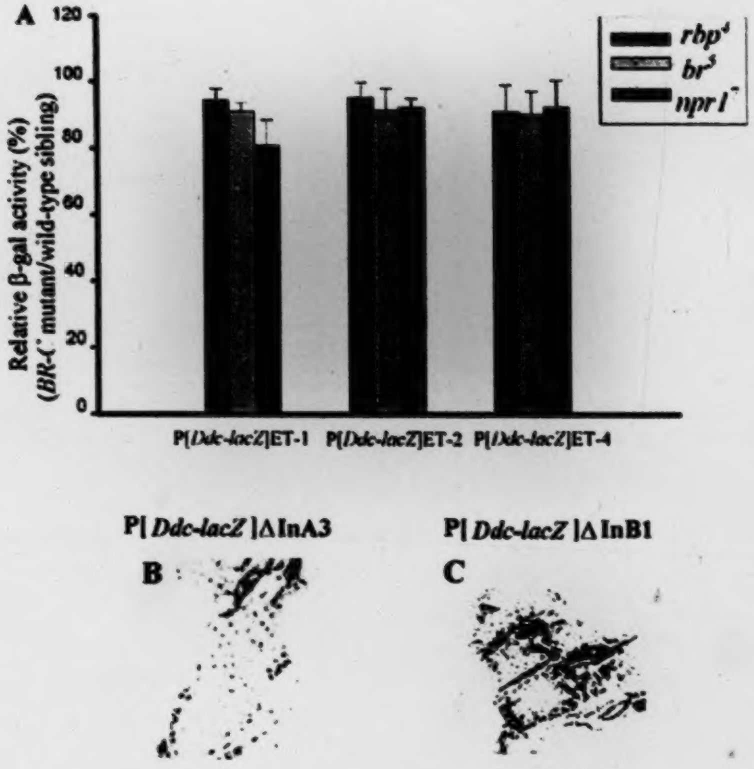

Figure 3.2. A. Effects of *BR-C* mutations on β-gal activity in the larval epidermis of transgenic lines P[*Ddc-lacZ*]ET-1, 2 and 4 carrying the intact *Eco*RV-*Taq*I reporter construct. β-gal activity in mutant males is expressed as a percentage of the activity in the wild-type sibs that were segregating in the same cross. The vertical bars represent the mean value of two independent assays. Error bars indicate the range. B,C. Epidermal expression patterns of transgenic lines carrying P[*Ddc-lacZ*] reporter constructs extending downstream of –208 bp.

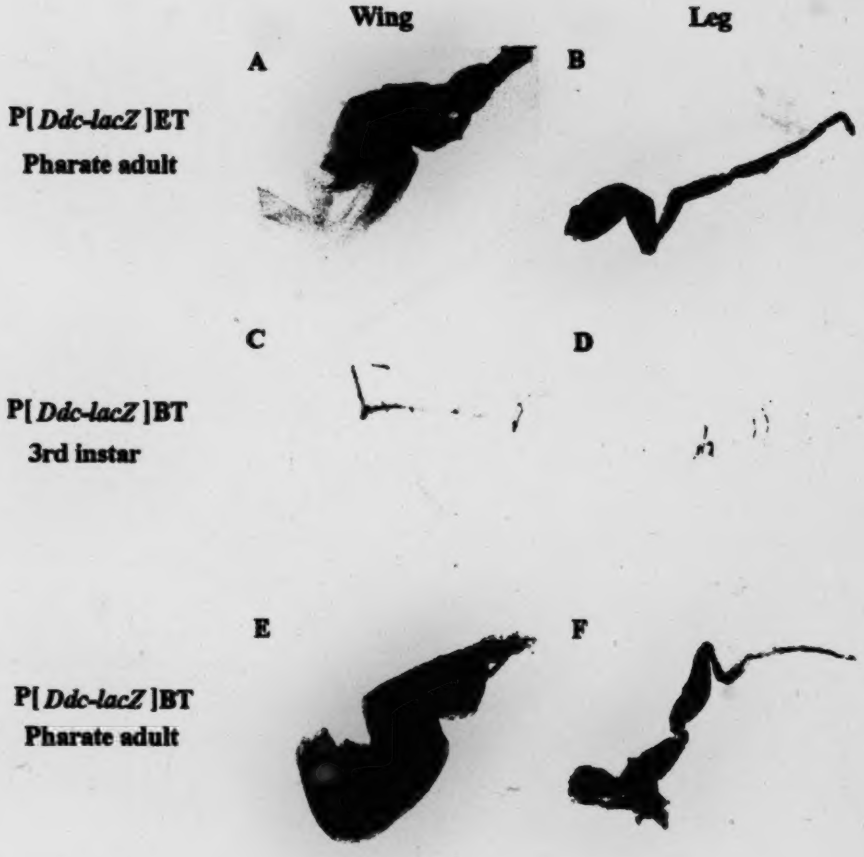
Wing
Leg
A
B
P[*Ddc-lacZ*]ET
Pharate adult
C
D
P[*Ddc-lacZ*]BT
3rd instar
E
F
P[*Ddc-lacZ*]BT
Pharate adult

Figure 3.3. Precocious *lacZ* staining in transgenic lines carrying P[*Ddc-lacZ*]ET and P[*Ddc-lacZ*]BT. Appendages (wing and leg) were taken from pharate adult flies 60 hours after puparium formation. Imaginal discs were from late wandering third larval instar of the P[*Ddc-lacZ*]BT-carrying line.

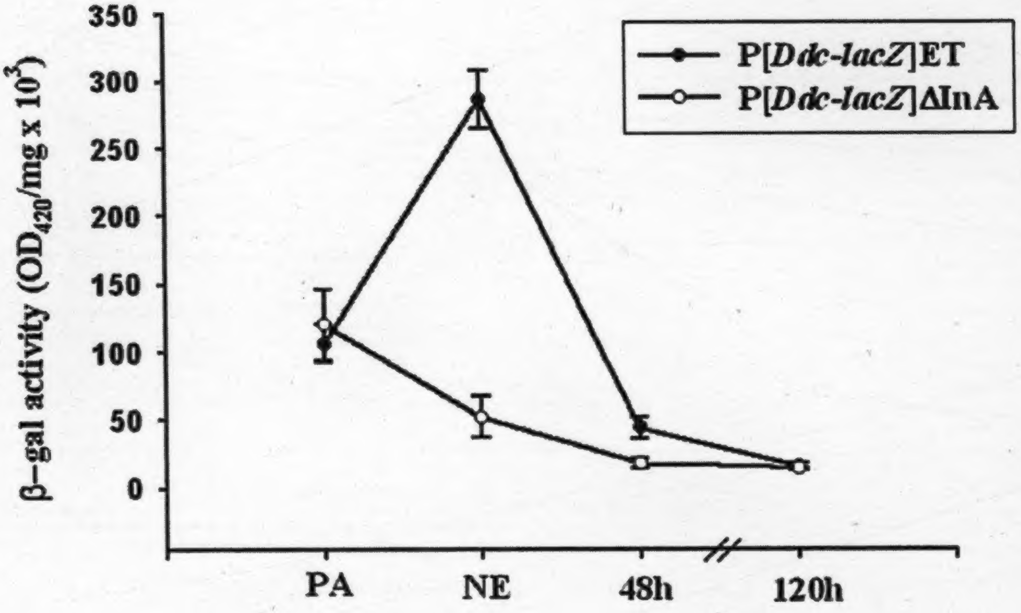

Figure 3.4. Effects of deleting the *BR-C* binding sites in the first *Ddc* intron on adult epidermal β-gal activity. The pharate adults in which eye pigmentation was visible (PA) and 0-1 hr newly eclosed (NE) and later adults were obtained from three independent lines of flies carrying either P[*Ddc-lacZ*]ET or P[*Ddc-lacZ*]ΔInA. The values represent the mean of two independent experiments. The error bars indicate the standard deviations from the means.

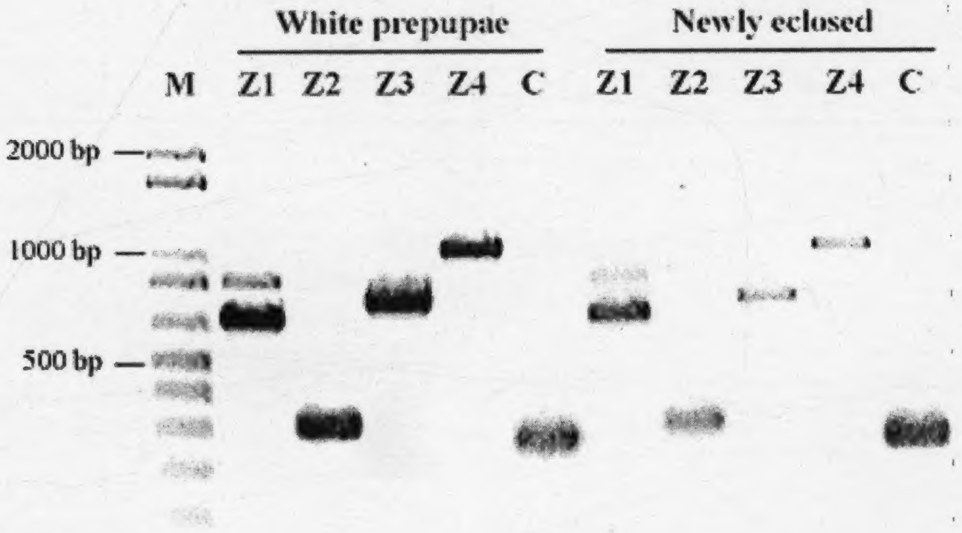
White prepupae
Newly eclosed
M
Z1
Z2
Z3
Z4
C
Z1
Z2
Z3
Z4
C
2000 bp
1000 bp
500 bp

Figure 3.5. RT-PCR analysis of *BR*-C$_{Z1\text{-}Z4}$ expression pattern in white prepupae and at eclosion. Total RNA was isolated from *yw* flies and reverse transcribed. The cDNA products were then amplified using the Z1-Z4 isoform specific primer pairs and visualized on a 2% agarose gel stained with ethidium bromide. M, 1kb ladder; C, *rp49* control. The top band in the Z1 lane is a spurious co-amplification product that we have determined arises from the *heavy vein* gene (data not shown).

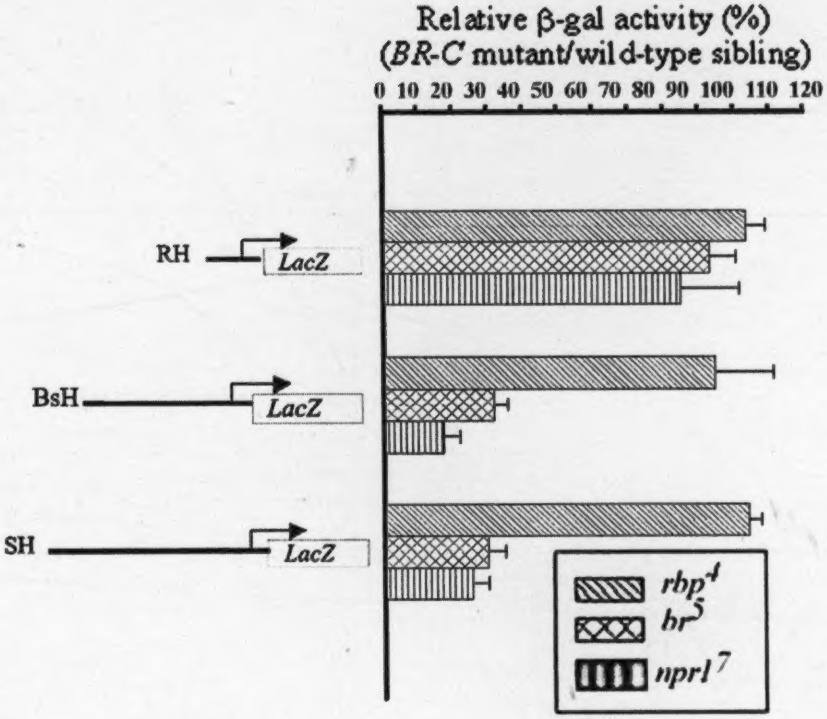

Figure 3.6. The effects of *BR-C* mutations on the expression of reporters driven by various upstream *Ddc* regions. β-gal activity in mutant males is expressed as a percentage of the activity in the wild-type sibs that were segregating in the same cross. The horizontal bars represent the mean value of two independent assays on two different transgenic strains. Error bars indicate the range.

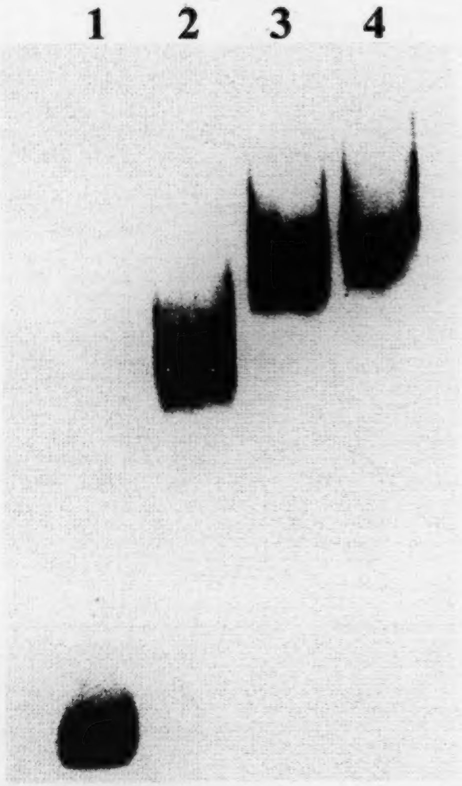

Figure 3.7. Mobility shift analysis using Z2. Binding of Z2 to the end-labeled 360 bp sub-fragment within the *Bsm*I-*Eco*RI region was carried out with 0, 50, 100, and 200 ng (lanes 1-4 respectively) of Z2 protein.

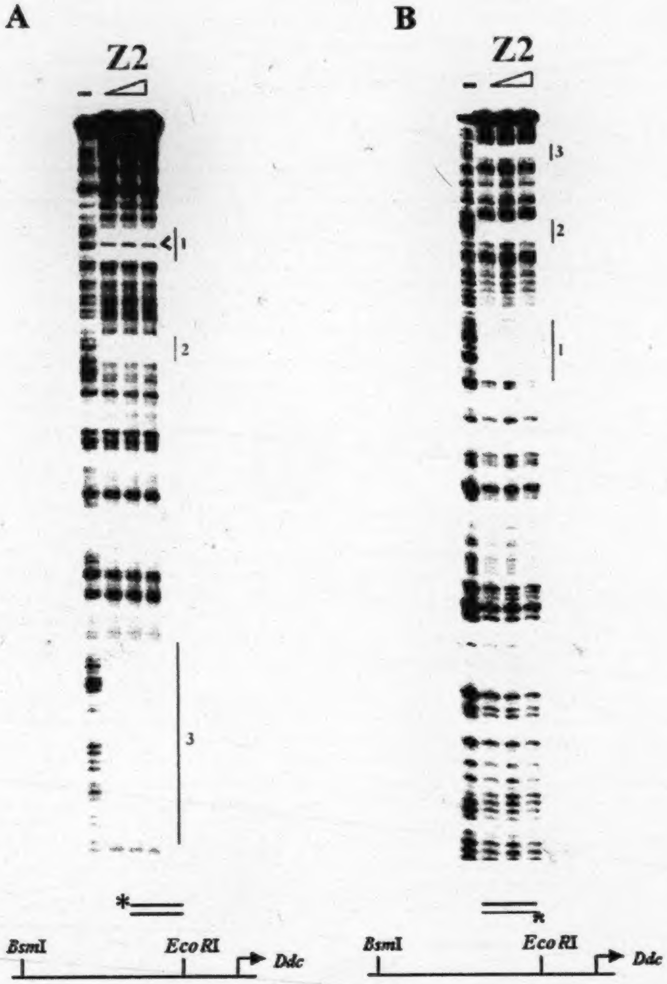

Figure 3.8. DNase I footprinting analysis of the 360 bp fragment using Z2. The coding (A) and non-coding (B) strand probes were digested in the absence (-) or presence of an amount of recombinant Z2 protein that was increased from 100 ng to 400 ng. The DNA probes are schematically indicated relative to the *Ddc* transcriptional start site at the bottom. The arrowhead marks the DNase I hypersensitive band within footprint 1.

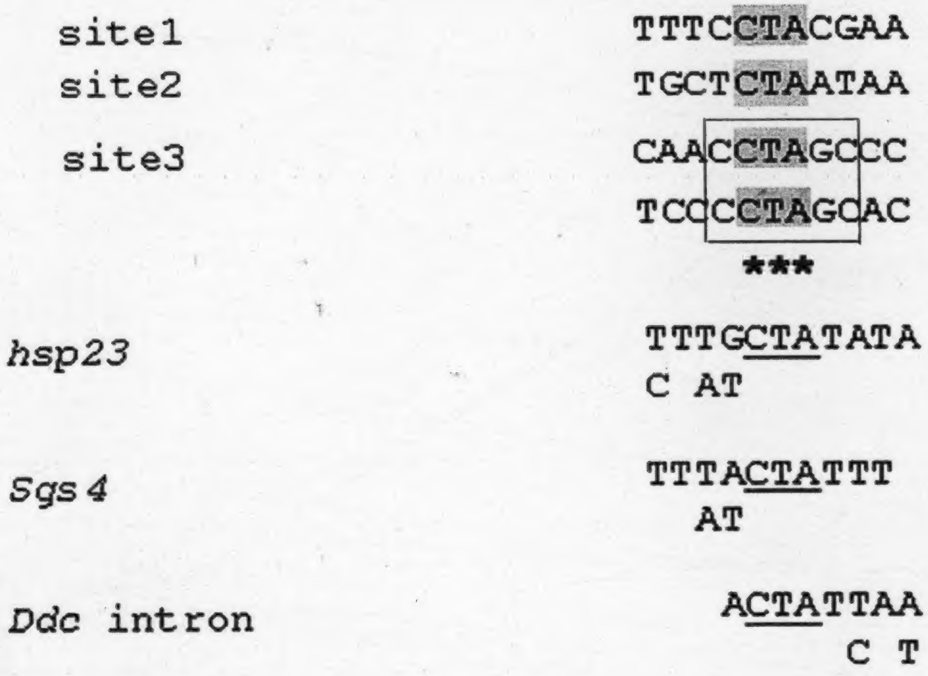
site1
TTTCCTACGAA
site2
TGCTCTAATAA
site3
CAACCTAGCCC
TCCCCTAGCAC
***
hsp23
TTTGCTATATA
C AT
Sgs 4
TTTACTATTT
AT
Ddc intron
ACTATTAA
C T

Figure 3.9. The three Z2 footprinting sequences are aligned with those determined for *Sgs4*, *hsp23*, and the *Ddc* intron. Asterisks mark the conserved tri-nucleotide core sequence. The box indicates the direct repeats.

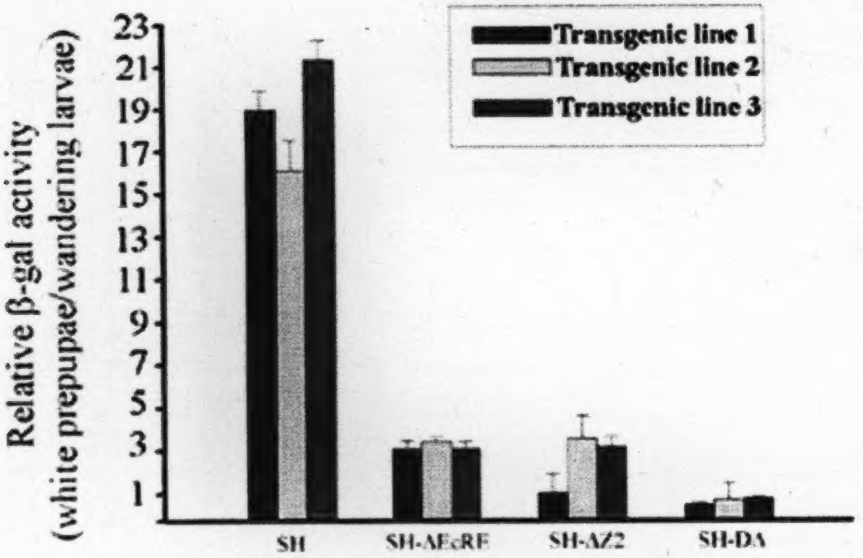

Figure 3.10. Activity of reporters carrying a deletion of EcRE$_{-97}$, the Z2 binding sites or both. Reporter activity in white prepupae is expressed as a ratio of the activity of the early wandering third instar larvae. The value shown in the diagram is the average of two assays for each independent transgenic line of the different constructs. The error bars indicate the range.

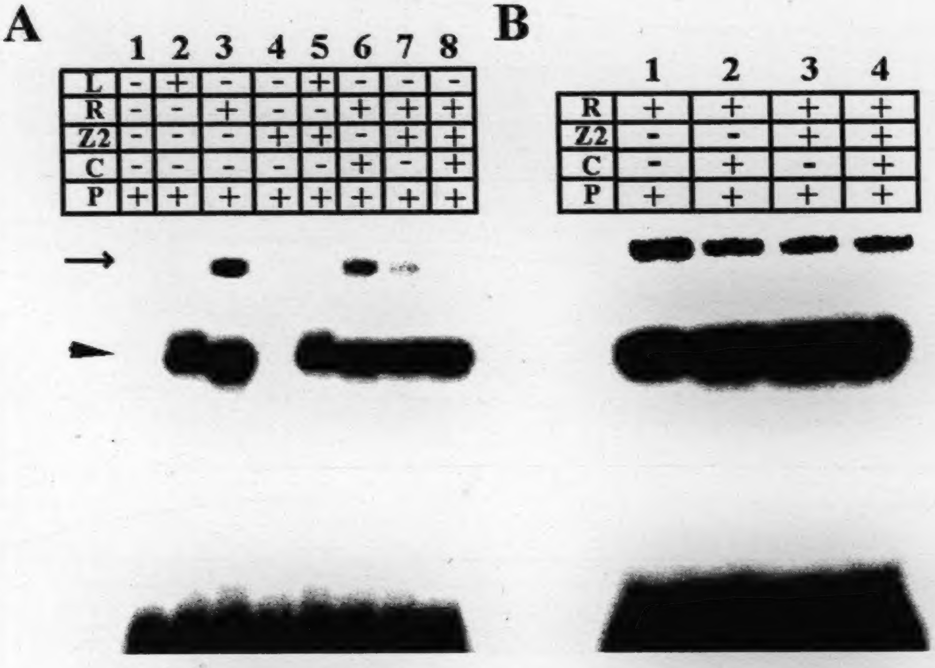

Figure 3.11. The effects of Z2 on the affinity of the EcR/USP receptor complex for EcRE$_{-97}$. A. Z2 protein (+/- the 360 bp fragment), was first incubated with the EcR/USP complex for half an hour before adding the EcRE$_{-97}$ probe to the binding reaction. B. The EcR/USP complex was first incubated with the EcRE$_{-97}$ probe for half an hour before the addition of the Z2 protein (+/- the 360 bp fragment). L: rabbit reticulocyte lysate; R: EcR/USP complex; C: 360 bp cold DNA fragment; P: end-labeled EcRE$_{-97}$ probe (Chen et al., 2002). The specific EcR/USP/ EcRE$_{-97}$ probe complex is indicated by the arrow. The arrowhead indicates a common non-specific band due to binding of a protein in the reticulocyte lysate.

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Chapter 4

Drosophila hormone receptor 38 may function as a repressor of *Ddc* during early metamorphosis

Introduction

The nuclear receptor superfamily is a family of transcription factors including the receptors for the steroids, retinoids, thyroid hormones, and vitamin D along with a large number of related proteins, the orphan receptors, for which no ligand is known (Mangelsdorf et al., 1995). These structurally related proteins also have conserved molecular mechanisms for receptor function throughout the evolution of higher animals. Studies done in *Drosophila melanogaster* have established a role for members of the nuclear receptor superfamily in numerous aspects of embryonic and post-embryonic development (Thummel, 1995). To date, 16 genes of the nuclear receptor superfamily have been isolated in Drosophila, all encoding members of the heterodimeric and orphan classes of receptors. In particular, half of the known Drosophila superfamily members, *EcR, E75, E78, βFTZ-F1, DHR3, DHR39, DHR78,* and *DHR96,* are regulated by the steroid hormone ecdysone (Ayer et al., 1993; Fisk and Thummel, 1995; Koelle et al., 1991; Kozlova et al., 1998; Lam et al., 1999; Segraves and Hogness, 1990; Stone and Thummel, 1993; Woodard et al., 1994). Remarkably, *EcR* is the only known ligand-dependent nuclear receptor identified to date in Drosophila. It acts as the ecdysone receptor upon heterodimerization with Ultraspiracle (USP) (Thomas et al., 1993; Yao et al., 1993). The rest of the seven receptors belong to the orphan receptor class and appear to contribute significantly to the complex developmental pathways associated with metamorphosis. Among them, *DHR78* functions at the top of the mid-third instar regulatory hierarchy, preparing the animal for puparium formation in response to the high titer late larval pulse of ecdysone (Fisk and Thummel, 1998). *DHR3* functions as a switch that defines the larval-prepupal transition by arresting the early regulatory response to ecdysone at puparium formation and facilitating the induction of *βFTZ-F1* in mid-prepupae through a precisely controlled timing mechanism that is dependent on the disappearance of E75B repressor from *βFTZ-F1* promoter (Lam et al., 1999; White et al., 1997). *βFTZ-F1* itself provides the early genes, such as *BR-C*, *E74*, and *E75*, with the competence to be re-induced by ecdysone in late prepupae (Woodard et al., 1994).

Recent functional studies of Drosophila orphan receptors have indicated that these factors might also interfere with ecdysone signaling pathways by either binding to EcREs or forming inactive heterodimers with EcR or USP. In spite of intensive efforts to search for such interactions occurring in Drosophila, however, so far only two alternate heterodimers have been discovered. *Seven-up*, the Drosophila homolog of the chicken ovalbumin upstream transcription factor (COUP-TF), negatively regulates hormonal signaling involving USP in a tissue culture system (Zelhof et al., 1995). Ectopic expression of *Svp* in developing animals during a narrow period of time leads to lethality and the dead animals have a terminal phenotype similar to that exhibited by animals that either fail to make ecdysone or lack a key component of the ecdysone receptor complex (Zelhof et al., 1995). Concomitant over-expression of *USP* rescues the larvae from the lethal effects of *Svp*, suggesting functionally that *Svp* is impinging on a USP-based signaling pathway. As demonstrated with use of the yeast two-hybrid system, an interaction between Svp and EcR is readily observed, while no interaction between Svp and USP is detected. A reasonable interpretation of these results suggests that Svp inhibits EcR-USP by formation of an inactive heterodimer. USP's second partner is hormone receptor 38 (DHR38), a homolog of the vertebrate nerve growth factor-induced protein B (NGFI-B)/Nurr1/Nor1 orphan receptors (Fisk and Thummel, 1995). Transfection experiments in Schneider cells show that DHR38 can compete with EcR for dimerization with USP and consequently disrupt ecdysone-dependent transcription (Sutherland et al., 1995). However, the best evidence supporting such an interaction in normal development comes from the mosquito *Aedes aegypti* (Zhu et al., 2000). In these so-called anautogenous mosquitoes, vitellogenesis, the key event in egg maturation, is initiated only after a female mosquito ingests vertebrate blood. A blood meal triggers a hormonal cascade with ecdysone as the terminal signal, which in turn activates yolk protein precursor (*YPP*) genes in the metabolic tissue, the fat body. An important adaptation for anautogenicity is the previtellogenic developmental arrest (the state-of-arrest) preventing the activation of *YPP* genes in previtellogenic females prior to blood feeding. Co-immunoprecipitation experiments indicate that in the fat body of a previtellogenic female mosquito during the state-of-arrest, AaUSP predominantly exists in a complex with AHR38 and not with AaEcR. On the contrary, in the fat body of vitellogenic female mosquitoes AaUSP is able to form a complex with AaEcR. Thus, AHR38 seems to act as a blocking agent of the ecdysone signaling during the previtellogenic arrest via dimerization with AaUSP.

In this chapter we report that the orphan receptor DHR38, as a monomer, binds to a silencing element within the Dopa decarboxylase (*Ddc*) gene 5' upstream regulatory region. It might function as a repressor to prevent *Ddc* from precocious expression in both epidermal and most importantly, in the imaginal discs, prior to the sclerotization pathway being activated later in development. This is the first evidence that in Drosophila, *DHR38* as it has been shown in the tissue culture system and in the mosquito *Aedes aegypti*, could negatively regulate the ecdysone signaling pathway, although the underlying mechanism might be distinctly different.

Results

Identification of a silencing element required to repress Ddc

From previous work on transformants carrying deletions within the 5'-*Ddc* region (see Chapter 2), it was concluded that a silencing element, located between -2067 and –1427 bp, maintained *Ddc* at a low level during third instar. Loss of this silencing element resulted in precocious epidermal *Ddc* expression in early wandering stage larvae. In order to further define the silencing element, reporter constructs were made in which three overlapping sequences from -2067 and –1427 bp, each about 200 bp long, were inserted immediately upstream of the *Eco*RI-*Hpa*I fragment (P[*Ddc-lacZ*]ΔSi series, Fig. 4.1) to determine which of these sub-fragments could repress the premature expression. Whereas all of the transformant lines carrying either P[*Ddc-lacZ*]ΔSi-1 or P[*Ddc-lacZ*]ΔSi-2 showed precocious β-galactosidase activity in both the larval epidermis (Fig. 4.2A, E) and in the imaginal discs (Fig. 4.2B-D and F-H), the two lines carrying P[*Ddc-lacZ*]ΔSi-3 exhibited very little hypodermal staining in early wandering larvae (Fig. 4.2I) and no disc staining (Fig. 4.2J-L). Thus, the silencing element is mapped within the 219 bp region between -1645 and –1427 bp defined by the ΔSi-3 deletion.

The silencing element contains a DHR38 *binding site*

In order to identify the nature of the repressor that acts through the 219 bp silencing element, we did a blast search of the known transcription factor binding sites. An AAT sequence followed by a steroid hormone response element half-site AGGTCA on the non-transcribed strand (Fig. 4.3), elicited particular interest. This nine bp sequence bears considerable similarity with the binding site of the vertebrate orphan receptor nerve growth factor-induced protein B (NGFI-B). In fact, the only mismatch is a T instead of A immediately 5' to the half-site sequence. The Drosophila homologue of NGFI-B, *Dhr38,* has been identified and shown to repress ecdysone-induced transcription of a reporter gene in Schneider cells (Fisk and Thummel, 1995; Sutherland et al., 1995). To test whether DHR38 protein can bind to the nine bp site identified within the silencing element, an end-labeled oligonucleotide probe containing the AATAGGTCA site (shaded in Fig. 4.3) was used in an electrophoretic mobility shift assay (EMSA). This probe showed extensive retardation by DHR38 (Fig. 4.4, lanes 2-4) and the binding to this 28 bp probe was eliminated in the presence of a 50-fold excess of unlabelled competitor (Fig. 4.4, lane 5).

A favored model to interpret the silencing effect of DHR38 is the hindrance of an ecdysone signaling pathway via heterodimerization with USP (Sutherland et al., 1995). To assess the possibility that DHR38 interacts physically with USP, the 219 bp silencing element was end-labeled and used in EMSA. As shown in Figure 4.5, USP alone did not bind to this region (lane 3). When both DHR38 and USP were incubated with the probe, no new complex was observed (compare lane 4 with lane 2). Thus, DHR38 alone bound to the silencing element as a monomer.

Deletion of the DHR38 *binding site mimics the deletion of the silencing element*

In order to confirm the functional importance of the DHR38 binding site, a deletion that specifically removed the 9 bp sequence (5'-AATAGGTCA-3') was introduced into the P[*Ddc-lacZ*]SH-ΔBR construct. Three transformed lines carrying the P[Ddc-lacZ] ΔHR38 deletion construct were generated and early wandering larvae from one independent lines were stained for premature β-galactosidase activity. Strong *lacZ* staining in the epidermis and in the leg discs can be detected (Fig. 4.6 D and E) while the eye disc (Fig. 4.6 F) and wing disc (data not shown) showed relatively little staining. This result is very similar to the staining pattern of early wandering larvae of flies carrying the silencing element deletions, such as P[*Ddc-lacZ*]BsH and P[*Ddc-lacZ*]RH (Chen et al., 2002) despite the fact that the staining pattern of imaginal discs varies considerably from one line to another.

A Dhr38 mutant shows premature Ddc expression

A null mutation of *DHR38* resulted in high level of *Ddc* expression when *Ddc* transcription levels were compared with the wild type siblings at early wandering stage about 18 hr before puparium formation (T. Kozlova, personal communication). Since this null allele was not available at the time, we used an EMS induced allele of *DHR38*, *DHR38*^{56} for our test. This allele behaves as a pupal lethal in combination with the chromosomal deletion, *Df(2)Ketel*^{RX32} (Kozlova et al., 1998). A P[*Ddc-lacZ*]SH reporter line, which carries the *Sca*I-*Hpa*I fragment (Fig. 4.1), was combined with the *DHR38*^{56} allele. *yw P[Ddc-lacZ]SH; Dhr38*^{56}*/CyO,y*$^{+}$ male flies were mated to virgin females *yw; RX*^{32}*/CyO,y*$^{+}$. Early wandering stage female larvae were selected for staining and *lacZ* reporter activities in *RX*^{32}*/Dhr38*^{56} flies were compared with those in the *RX*^{32}*/CyO,y*$^{+}$ *or Dhr38*^{56}*/CyO,y*$^{+}$ siblings. Our preliminary result showed subtle differences in β-gal staining patterns between the mutant and wild type control siblings. The *y* female had sporadic staining in pieces of integument from both the anterior and posterior halves of the organism, whereas the *y*+ organism showed very little staining (data not shown). It must be noted that this is a very preliminary result and must be repeated. Currently, the cross to test the null allele of *Dhr38* is well underway.

Discussion

During Drosophila development, the larva, pupa, and adult are protected and supported by exoskeletons or cuticles that are secreted by the underlying epidermis. Because cuticles are comparatively inelastic structures, epidermal morphogenesis occurs only when the epidermis is unencumbered by an overlying cuticle. Thus, during metamorphosis, cellular and tissue morphogenesis occurs at two stages, before the deposition of the pupal cuticle and between the apolysis of the pupal cuticle and the formation of the adult cuticle. *Ddc*, which encodes dopa decarboxylase, an enzyme required in the tanning and hardening of the cuticle, is actively repressed during these two stages. Interestingly, stage-specific repression is mediated through *cis*-acting silencing domains located at different *Ddc* genomic regions, implying different repressors are involved. Here, we report that the larval stage repression may be a function of the orphan receptor *DHR38*.

DHR38 was first identified as the Drosophila homolog of a group of vertebrate orphan receptors typified by the nerve growth factor-induced protein B (NGFI-B) (Sutherland et al., 1995). Members of this group have been implicated as transcriptional regulators which exert their effects via binding to a DNA element, the NGFI-B response element (NBRE), AAAAGGTCA (Wilson et al., 1993; Zetterstrom et al., 1996). DHR38 binds specifically to the NBRE sequence, as expected for a homolog of NGFI-B (Fisk and Thummel, 1995). We show here, that despite a single base change in the 5' AAA flanking sequence, DHR38 still recognizes specifically the response element in the *Ddc* upstream region.

A notable feature of the NGFI-B family is the capacity to bind DNA as a monomer (Wilson et al., 1993). This binding ability has also been demonstrated for DHR38 (Fisk and Thummel, 1995). In addition to acting as monomers, nuclear receptors of the NGFI-B family can heterodimerize with RXR and bind to recognition response elements arranged as direct repeats of AGGTCA spaced by five nucleotide

(Perlmann and Jansson, 1995). RXR is a promiscuous nuclear receptor, which serves as a heterodimeric partner for the retinoic acid receptor (RAR), the thyroid hormone receptor (TR), the vitamin D receptor (VDR) and the fatty acid/peroxisome proliferator-activated receptor (PPAR) (Mangelsdorf et al., 1995; Mangelsdorf and Evans, 1995). Likewise, DHR38 can be an alternative partner of USP, the insect homolog of RXR, and an obligatory partner of EcR (Sutherland et al., 1995). DHR38 can compete *in vitro* with EcR for dimerization with USP and consequently disrupts EcR-USP binding to an EcRE. Transfection experiments in Schneider cells show that DHR38 can repress ecdysteroid-dependent transcriptional activation (Sutherland et al., 1995). Alternatively, both DHR38-USP and EcR-USP heterodimers can compete for binding to selected EcREs, as demonstrated in Drosophila *ng-1* and *ng-2* intermolt genes, containing directly repeated half-sites spaced by 12 bp (Crispi et al., 1998). Despite the clear evidence that in the anautogenous mosquito AHR38 plays an important function by blocking ecdysone responsiveness in the target tissues at the state-of-arrest, through heterodimerization with AaUSP, as well as by possibly competing for binding EcREs, the biological role of DHR38 in Drosophila remains obscure. We propose here that DHR38, not coupled with USP, acts alone to mediate the repression of *Ddc* during early third instar stage by binding to a relatively conserved response element. This result is consistent with our previous result showing USP is not involved in the repressive process (Chen et al., 2002). By using a *usp* cDNA under the control of a heat-inducible promoter to rescue *usp* mutants past their early lethal phase to the third instar stage (Hall and Thummel, 1998), we have shown that no apparent premature β-gal activity could be observed in the epidermal tissue or in any of the larval imaginal discs.

Although a precocious high level *Ddc* expression has been seen in a recently isolated null *DHR38* mutant (T. Kozlova, personal communication), the EMS induced *DHR38*^{56} allele did not show apparent high-level *Ddc* premature expression. This allele, was one of the four DHR38 mutant alleles available at the time when we started the experiment and shows the earliest lethality among the four alleles (Kozlova et al., 1998). Two thirds of the hemizygous animals develop normally until the late pupal stage (85-90 hr after pupariation, AP), and pharate adults look normal externally when dissected from pupal cases (Kozlova et al., 1998). However, when the pharate flies start moving shortly before eclosion (around 90 hr AP at 25 °C), the adult cuticle is ruptured followed by leakage of haemolymph and melanization within the pupal case. The phenotype suggests that this allele does not affect puparium formation and pupal cuticle formation; but affects adult cuticle formation, possibly leading to incomplete sclerotization.

Although disappointing at the moment, the result is perhaps not surprising. In terms of its large size and complex structure, the *DHR38* gene is a typical member of the steroid receptor superfamily in Drosophila (Kozlova et al., 1998). It spans at least 40 kb in length and includes four introns varying in size from 117 bp to more than 20 kb. At least four developmentally regulated mRNA species are detected, apparently generated by alternative promoters and polyadenylation sites. Of the two best characterized mRNA species, the one corresponding to 4.0 kb, the so called cTK61 transcript, is relatively more larval-specific while the one corresponding to a 5.0 kb cTK11 transcript shows the most dramatic enhancement in pupae and persists into the adult stage. The phenotype of *DHR38*^{56} suggests that it might carry a mutation in the cTK11 isoform. The best candidate so far for the *DHR38* isoform that performs the repressive function is cTK61 because it is expressed through the larval stages. It becomes notably reduced during 0-8 hr at pupariation and is again enhanced during 8-24 hr after puparium formation, corresponding well with the requirement to fulfill the function of the *Ddc* repressor.

In summary, we report here that a Drosophila orphan receptor, DHR38, binds alone to a conserved NBRE located within the silencing domain of *Ddc* 5' upstream region. It might function as a repressor to inhibit premature *Ddc* expression in the larval epidermal tissue and in the imaginal discs. Like most other members of the nuclear receptor superfamily in Drosophila, *DHR38* encodes multiple isoforms and each isoform performs different functions throughout Drosophila development. Final confirmation depends on further characterization of *DHR38* and elucidation of the nature of the *DHR38* mutant alleles at the molecular level. Currently we are setting up crosses to test the null allele of *DHR38*. Also we are conducting heat shock induced ectopic expression of cTK61 and CTK11 at pupariation in order to clarify which DHR38 isoform is required to mediate the repression.

Materials and Methods

Drosophila crosses

Stocks used in this study were maintained at 25°C on a standard cornmeal/molasses medium. The *Dhr38* mutations were kindly provided by Dr. Kozlova (Kozlova et al., 1998). The late pupal lethal $Dhr38^{56}$ and the deficiency *Df(2)ketelRX32* (abbreviated as *RX32*) were used in this study. *yw P[Ddc-lacZ]SH;* $Dhr38^{56}/CyO,y^{+}$ male flies were mated to virgin females *yw;* $RX^{32}/CyO,y^{+}$. $RX^{32}/Dhr38^{56}$ flies were distinguished from the $RX^{32}/CyO,y^{+}$ or $Dhr38^{56}/CyO,y^{+}$ by the absence of y^{+} marker.

Plasmid construction

The extent of the *Ddc* region driving *lacZ* expression in the *lacZ* reporter plasmids used in this study is shown in Fig. 4.1. All plasmids used in this paper were assembled from pPelican, a derivative of p[CaSpeR-AUG-*β*–gal] that carries a *gypsy* insulator on either side of the *lacZ* reporter gene (Barolo et al., 2000). Into this vector, we cloned fragments from the *Ddc* genomic region that included its transcription start site. The assembly of P[*Ddc-lacZ*]SH-ΔBR has been described elsewhere (see Chapter 3). To make the ΔSi deletion series, PCR products were amplified by *Pfu* (Stratagene) using the following primer pairs: for P[*Ddc-lacZ*]ΔSi-1, 5'-CTTTTCGATTTTGTTTCTGC-3' (-2067 to –2048 bp) and 5'-GCCGATCTT-AAGGACCAAT-3' (-1828 to –1847 bp); for P[*Ddc-lacZ*]ΔSi-2, 5'-GCTGAATTGGTCCTTAAGA-3' (-1851 to –1833 bp)and 5'-

CGATCTGTAATTAATTGGG-3’ (-1625 to –1643 bp); for P[*Ddc-lacZ*]ΔSi-3, 5’-TTCCCAATTAATTACAGATCG-3’ (-1645 to –1625 bp) and 5’-AAGTATTCTCCGCTTATAGG-3’ (-1427 to –1446 bp). The products were inserted into the *Sma*I site of pBluescript SK(+) and subsequently re-cloned into P[*Ddc-lacZ*]RH as *Xba*I-*EcoR*I fragments. The desired orientations were confirmed by PCR.

The reporter plasmid carrying the DHR38 binding site deletion within the *Sca*I-*Bsm*I fragment (P[*Ddc-lacZ*]ΔHr38) was created by inverse PCR using the SH-ΔBR insert in pBluescript SK(+) as the template (see Chapter 3) and primers: 5'-CGTATTTAGTCTGAATCTATATG-3' (-1569 to –1591 bp) and 5'-GAAGCTCAGCGATGTGATGT-3' (-1559 to -1540 bp). The desired fragment was released from this plasmid as a *Pst*I-*Kpn*I fragment and subcloned into pPelican.

Electrophoretic mobility shift assay

DHR38 and USP proteins were kindly provided by Dr. Henry Krause. Mobility shift assays were carried out as described in Wilson et al. (1991).An oligonucleotide carrying the putative DHR38 binding site in *Ddc* was obtained by annealing the single stranded sequences 5’-CTAAATACGTGACCTATTGAAGCTCAGC-3’and 5’-TCGCTGA-GCTTCAATAGGTCACGTATTT-3’ (the binding site is underlined). DHR38 and/or USP proteins up to 500 ng were incubated with 1 ng of ^{32}P-end-labelled DNA probe in the presence of 2 μg of non-specific competitor poly(dI-dC) (Pharmacia), in buffer that was 75 mM NaCl, 5% glycerol, and 0.5 mM DTT, and 1 mM EDTA. The incubation was carried out in 20 μl at 22 °C for 30 min and was followed by loading on a 4% non-denaturing polyacrylamide gel in a 0.5X TBE running buffer. After electrophoresis, the gel was dried for autoradiography.

Germline transformations and histochemical assays

Microinjections and staining of the integument and the imaginal discs for *lacZ* activity were carried out as described elsewhere (Chen et al., 2002).

Acknowledgments

I wish to thank Dr. Henry Krause for the DHR38 and USP proteins, Dr. Ping Yang for the transgenic flies carrying DHR38 binding site deletion, Dr. Tatiana Kozlova for the *Dhr38* mutant stocks, and Scott Hanna for microinjection. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

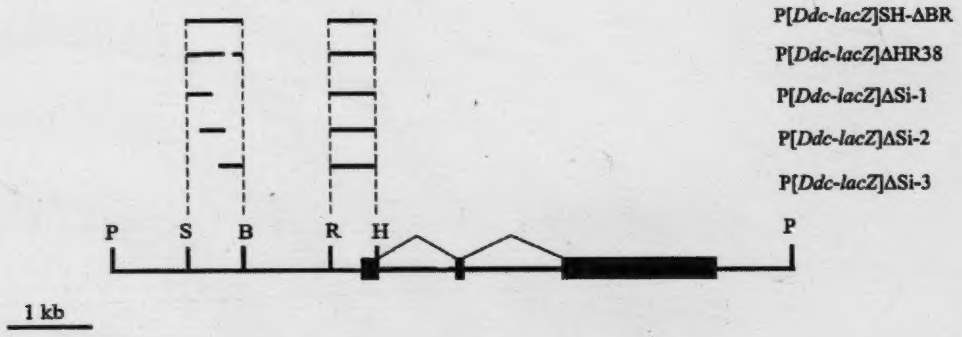
P[*Ddc-lacZ*]SH-ΔBR
P[*Ddc-lacZ*]ΔHR38
P[*Ddc-lacZ*]ΔSi-1
P[*Ddc-lacZ*]ΔSi-2
P[*Ddc-lacZ*]ΔSi-3
P
S
B
R
H
P
1 kb

Figure 4.1. Schematic diagram of the P[*Ddc-lacZ*] reporter constructs used in this paper. The epidermal-specific splicing pattern of the *Ddc* transcription unit is positioned on a restriction map of the 7.6 bp *Pst*I-*Pst*I genomic fragment that is sufficient for normal *Ddc* developmental expression. The sites shown are *Bsm*I (B), *Eco*RI (R), *Hap*I (H), *Pst*I (P), and *Sca*I (S).

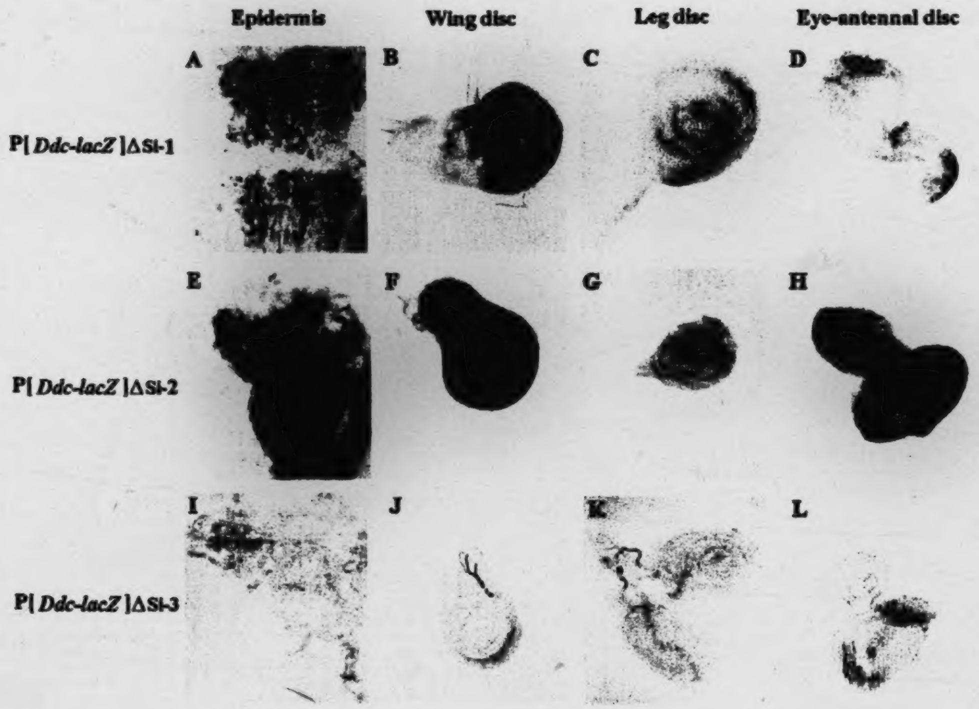
Epidermis
Wing disc
Leg disc
Eye-antennal disc
A
B
C
D
P[Ddc-lacZ]ΔSi-1
E
F
G
H
P[Ddc-lacZ]ΔSi-2
I
J
K
L
P[Ddc-lacZ]ΔSi-3

Figure 4.2. Epidermal expression patterns of transgenic lines carrying P[*Ddc-lacZ*]ΔSi reporter constructs. Tissues were obtained from early wandering third instar larvae. The integument (panels A, E, I), wing (B, F, J), leg (C,G, K) and eye-antennal (D,H, L) discs were stained for two hours to visualize β-galactosidase expression.

5′-TTCCCAATTAATTACAGATCGATCCTAAAACGAATCTAATCAC
TTGCCCATATCATATAGATTCAGACTAAATACGTGACCTATTGAAG
CTCAGCGATGTGATGTGTACACCAAACACCCGCTCGTTTATCTCTG
CCCTTGTTTACCCCATATGATGCCTGTTTATGCAATCCCCCTCTCA
AAGGCGCCATTCGACCCCTATAAGCGGAGAATACTT-3′

NBRE	AAAAGGTCA
DHR38	AATAGGTCA

Figure 4.3. The putative DHR38 binding site is aligned with the NGFI-B response element (NBRE). The box indicates the half-site. The oligonucleotide sequence used in the EMSA is shaded in the 219 bp silencing element.

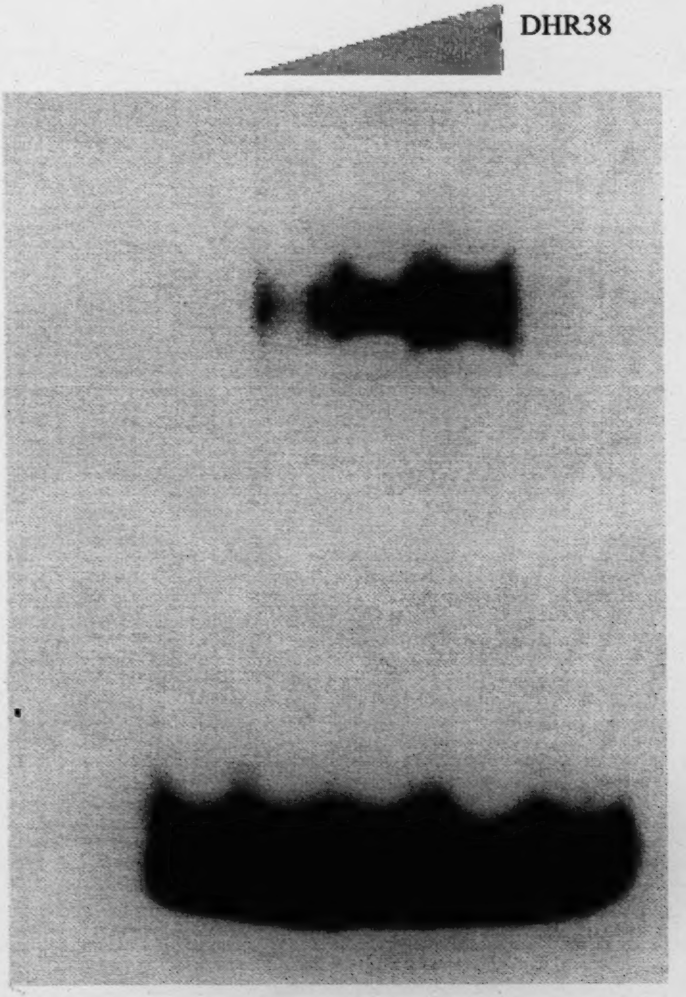

Figure 4.4. Electrophoretic mobility shift analysis using DHR38. Binding of DHR38 to the ^{32}P end-labeled oligonucleotide within the 219 silencing element region was carried out with 0, 100, 250, and 500 ng (lanes 1-4 respectively) of DHR38 protein. Lane 5 is the same as lane 4 except with the addition of 50 fold excess of unlabeled oligonucleotide DNA.

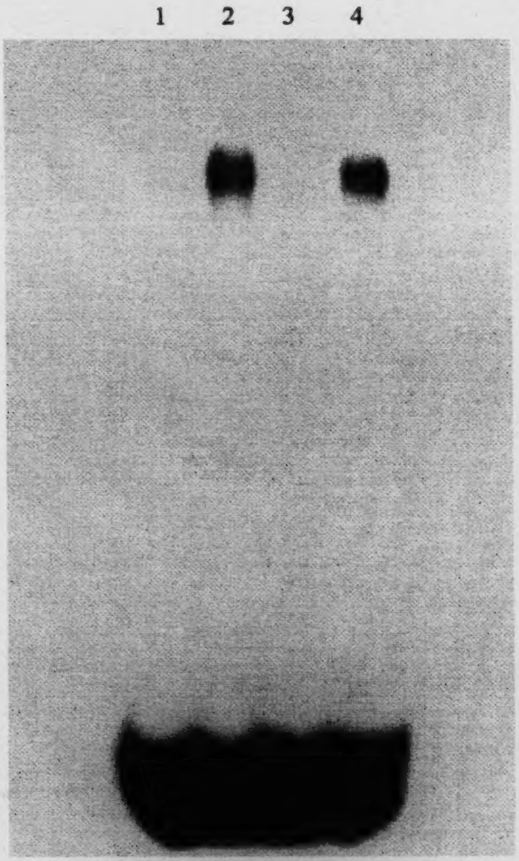

Figure 4.5. Electrophoretic mobility shift analysis using DHR38 and/or USP. Binding of DHR38 to the ^{32}P end-labeled 219 bp silencing element was carried out with 0, and 200 ng (lanes 1-2 respectively) of DHR38 protein. USP (200 ng) instead of DHR38 was added to the reaction in lane 3 and both DHR38 and USP were added in lane 4.

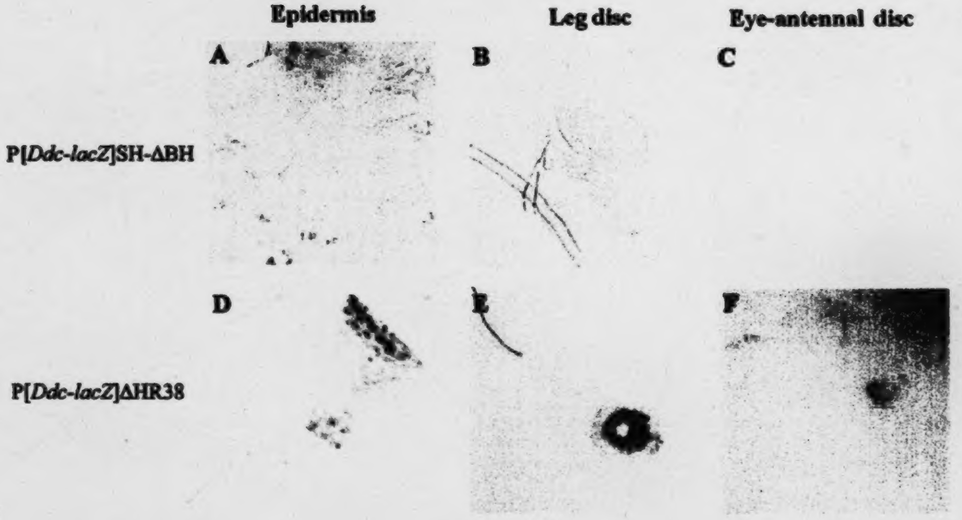
Epidermis
Leg disc
Eye-antennal disc
A
B
C
P[*Ddc-lacZ*]SH-ΔBH
D
E
F
P[*Ddc-lacZ*]ΔHR38

Figure 4.6. Epidermal expression patterns of transgenic lines carrying wild-type and DHR38 binding site deletion constructs. Tissues were obtained from early wandering third instar larvae. The integument (panels A, E,), wing (B, F), leg (C,G) and eye-antennal (D,H) discs were stained for two hours to visualize β-galactosidase expression.

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Mangelsdorf, D., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R.M. (1995) The nuclear receptor superfamily: the second decade. Cell 83, 835-839.

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Chapter 5

Conclusions and future work

The work described in this thesis was primarily motivated by three key findings. Firstly, the feeding of 20-hydroxyecdysone to the temperature sensitive mutant *ecd*^{1} larvae at 29 °C causes DDC activity to appear in the epidermis within 4 hr (Clark et al., 1986). Translatable mRNA is detected as early as 2 hr after feeding, which is comparable to the early puff induction in salivary gland chromosomes (Huet et al., 1995). Secondly, mutations in the *br* subcomplementation group of *BR-C* substantially reduces the levels of *Ddc* in the epidermis of mature third instar larvae (O'Keefe et al., 1995). Thirdly, a cluster of Z2 finger binding sites was identified in the first intron of the *Ddc* gene (Hodgetts et al., 1995). At the time, these sites were thought to mediate the up-regulation of epidermal *Ddc* at pupariation by *BR-C*. Taken together, these experiments strongly suggests that the epidermal expression profile of the *Ddc* gene is likely that of a typical early-late gene. However, no direct role for EcR/USP had been demonstrated for *Ddc* (or any other early-late genes) nor has a functional test of the intronic sites been made. The data contained in this thesis have established:

(1) The EcR-B1/USP heterodimer directly up-regulates *Ddc* expression at the end of larval life by binding to an imperfect ecdysone response element, EcRE$_{-97}$, located at position –97 to –83 bp relative to the transcription initiation site. This is the first report of an EcRE associated with an early-late gene in *Drosophila melanogaster*.

(2) At pupariation, *BR-C* acts synergistically with the ecdysone receptor to up-regulate *Ddc*. DNase I footprinting has identified four binding sites of the predominant Z2 isoform of *BR-C* within a distal regulatory element (-383 to –743 bp) that is required for maximal *Ddc* activity.

(3) The intronic BR-C binding sites are required for *Ddc* expression at eclosion. This is the first evidence to suggest that this important regulator of the early metamorphic events controlled by ecdysone is also involved in regulating target gene expression at the end of metamorphosis.

(4) At both pupariation and eclosion, *Ddc* is released from active silencing mechanisms that operate through distinct *cis*-acting regions of the *Ddc* genomic domain. A silencing domain between –1427 and –1645 bp represses *Ddc* in the imaginal discs during larval stages and a yet to be defined downstream silencing domain mediates the repression during early pupal stage.

(5) The Drosophila orphan receptor, DHR38, binds alone to a conserved NBRE located within the silencing domain of the *Ddc* 5' upstream region. One of its encoded multiple isoforms may function as a repressor to inhibit premature *Ddc* expression in the imaginal discs.

A model in which *BR-C* plays a key role in connecting the hormone signal and activation of *Ddc* is proposed and presented in Figure 5.1. In such a model, active repression by DHR38 and an unknown factor during early third instar and the early pupal stages respectively would keep *Ddc* expression in the epidermal tissue at a low basal level, thus allowing cell and tissue metamorphosis to occur without the restriction of an inelastic overlying cuticle. At pupariation, BR-C and the ecdysone receptor act together to release the *Ddc* gene from the repression incurred by DHR38 in the larval epidermal tissue, resulting in a high early-late response. DHR38 repression persists in the imaginal discs, which are destined to become the adult epidermis, until about 8 hr after puparium formation. Likewise BR-C would transduce the action of eclosion hormone before the last ecdysis, to further elevate *Ddc* expression that has been released from an unknown pharate adult stage-specific repressor protein. The exact mode of how the repression is relieved is not clear at this moment. However, in both cases, a drop from a high ecdysone titer seems to be a necessity for the de-repression. Even though the ecdysone titer is low during the late pupal stage, it is still high enough to be able to suppress developmental processes (J. Truman, personal communication). As the titer declines towards the end of adult development, developmental processes like cuticular pigmentation, molting fluid activation, ecdysis competence, post-ecdysis cell death are sequentially released from suppression.

Throughout this thesis, I have favored models in which the regulatory function of *BR-C* is mediated by a specific DNA binding process. Two modes of action, either as a transcriptional factor or chromatin structure modulator, are possible for the BR-C protein (Crossgrove et al., 1996; Dubrovsky et al., 2001; Jiang et al., 2000; Renault et al., 2001; von Kalm et al., 1994). Although we have not yet directly demonstrated a functional requirement of those BR-C binding sites identified in two potentially regulatory regions of the *Ddc* gene, DNAse I footprinting revealed that they all share a perfect conserved triplet core sequence with those present at other *BR-C* regulated genes. Given the fact that the BTB/POZ domain mediates a functionally relevant dimerization *in vivo*, promoting strong cooperative DNA binding to multiple sites (Bardwell and Treisman, 1994), it is attractive to propose that BTB/POZ domains are likely to play a critical role in targeting proteins to a cluster of specific sites. In this context, a physical interaction between the ecdysone receptor and the BR-C Z2 protein as deduced from the gel shift assay (Fig. 3.11) strongly suggests that a direct synergistic interaction between BR-C and ecdysone receptor complex exists *in vivo*.

Three major questions could be addressed in future studies.

(1) Which BR-C isoform functions at eclosion?

Genetic data show the Z2 isoform is responsible for *Ddc* induction at pupariation (Hodgetts et al., 1995; O'Keefe et al., 1995). With the evidence that *BR-C* also mediates important cellular functions during eclosion, a crucial next step is identifying the specific isoform in this process. *In vitro* and *in vivo* studies on the temporal *BR-C* isoform expression patterns suggest that the Z1 isoform is the best candidate (Bayer et al., 1996; Emery et al., 1994). In all these studies, a precipitous switch from Z2 to Z1 occurs. While these studies hint that the Z1 isoform is a possible *Ddc* regulator during eclosion, the ultimate *in vivo* proof must come from genetic experiments. Since flies that are mutant with respect to the four subcomplementation *BR-C* groups do not survive to eclosion, the best method to use is the RNAi technique (Lam and Thummel, 2000). When expressed under the control of a heat-inducible promoter, dsRNA can interfere efficiently and specifically with gene expression during a later stage of Drosophila development. Eclosion-specific expression of various BR-C isoform dsRNAs would allow us to determine which isoform is required for up-regulation of *Ddc* at eclosion. In the meantime, such studies will provide the ultimate proof of whether *BR-C* is indeed involved in this process. If true, the next question to explore is what factor signals the *BR-C* activation. In insects, ecdysis is thought to be controlled by the interaction between peptide hormones, in particular between ecdysis-triggering hormone (ETH) from the periphery and eclosion hormone (EH) and crustacean cardioactive peptide (CCAP) from the central nervous system (Truman, 1996). The final release of EH normally occurs approximately 40 min before ecdysis (Baker et al., 1999). The EH-producing neuron cell knockout flies generated by using an EH cell-specific enhancer to activate cell death genes *reaper* and *head involution defective* to ablate the EH cells (McNabb et al., 1997) would allow us to test whether *BR-C* expression is affected in these flies.

(2) Which DHR38 isoform functions as the repressor?

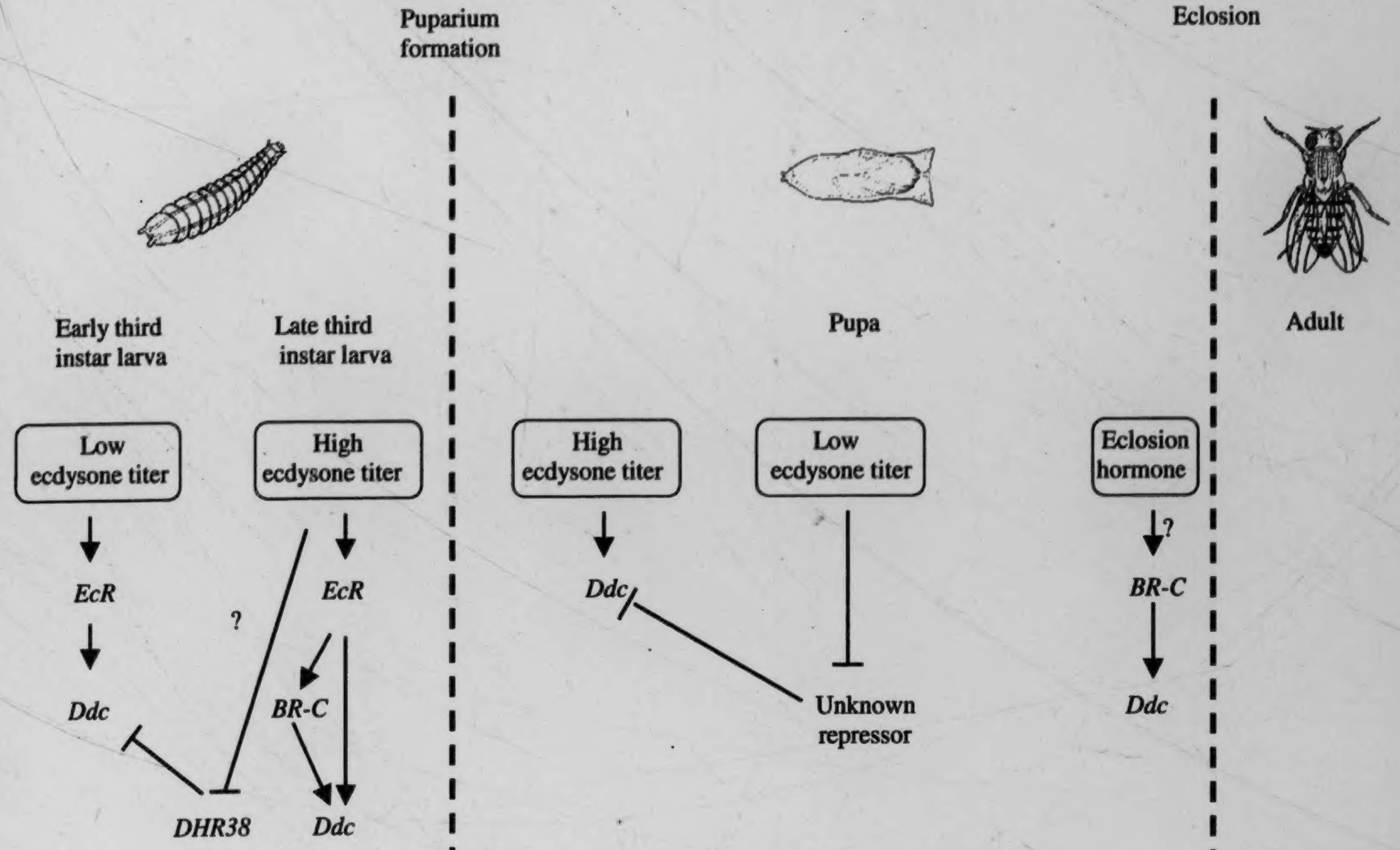
Three experiments are currently underway to confirm a repressive role for *DHR38*. Determining which isoform of DHR38 acts as the repressor may not be straightforward. As discussed in the Chapter 4, although the best candidate to date is cTK61, we cannot rule out the possibility that a different unidentified isoform is responsible for this function simply because of the complexity of the *DHR38* gene. If *Ddc* expression were relieved from the repression of a *DHR38* isoform, ectopic overexpression of this specific isoform would reversibly inhibit the *Ddc* expression at pupariation. Heat shock inducible cTK61 and cTK11 cDNA would help us to clarify this issue. Although a clear de-repression pattern has been shown in a newly isolated

Dhr38 null mutation by a Northern test in Dr. Kozlova's study (T. Kozlova, personal communication), the final confirmation of the repressive role conducted by *DHR38* is pending on the test of this null allele of *DHR38* on the transgenic flies carrying the *Ddc* 5' upstream silencing domain driving a *lacZ* reporter, such as P[Ddc-lacZ]SH. A slot blot experiment to repeat Kozlova's result is in progress. Given the important roles played by nuclear orphan receptors (Fisk and Thummel, 1998; Lam et al., 1999; White et al., 1997; Woodard et al., 1994), it is definitely worthwhile to further pursue a much more detailed molecular characterization of the *DHR38* locus.

(3) What is the pharate adult stage repressor?

Refining the mapping of the silencing element located downstream of *Taq*I site is the first step towards identifying the pharate adult stage repressor. Transformants are being produced in which the second intron has been inserted upstream of the *Sca*I-*Hpa*I fragment in P[*Ddc-lacZ*]SH-ΔBR construct (Fig. 4.1) (R. Hodgetts, personal communication). Repressed reporter expression in the pharate adult stage seen with this construct will localize the *cis*-acting silencing domain to the second intron. Similarly, further downstream sequence, such as the 3'-UTR, will be tested if necessary. Smaller internal deletions can then be introduced and tested. This would eventually lead to an identification of a relatively short piece of DNA that would allow us to search for any possible transcription regulatory factor binding sites. During the last decade, studies have made significant progress toward understanding the multiple ecdysone-triggered regulatory hierarchies around the onset of Drosophila metamorphosis (Thummel, 1996; Thummel, 2002). In contrast, our understanding of how the actions of ecdysone, eclosion hormone, and other possible hormonal signals, including the sesquiterpenoid juvenile hormone (JH), are transduced during the pupal stage and eclosion remains extremely limited (Baker et al., 1999; Harmon et al., 1995; Riddiford, 1993). Thus, further elucidation of the nature of the pharate adult stage-specific *Ddc* repressor may ultimately establish an important paradigm for how ecdysone-regulated gene cascades are established during the later stages of Drosophila development.

Figure 5.1. Schematic drawing of a suggested model for the regulation of the *Ddc* gene during Drosophila metamorphosis. Red bars represent repressive effects and green arrows represent inductive effect.



Puparium
formation
Eclosion
Early third
instar larva
Late third
instar larva
Pupa
Adult
Low
ecdysone titer
High
ecdysone titer
High
ecdysone titer
Low
ecdysone titer
Eclosion
hormone
EcR
Ddc
?
EcR
BR-C
DHR38
Ddc
Ddc
Unknown
repressor
?
BR-C
Ddc

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